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14. ABSTRACT: The study was designed to identify clinically accessible molecular biomarkers of traumatic brain injury (TBI) prior to definitive clinical diagnosis. Specifically, these studies were meant to identify, characterize, and validate small noncoding (snc) RNA biomarker species whose content in peripheral blood mononuclear cells (PBMC) could help to identify TBI cases from the Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) veteran population. In initial Biomarker Discovery studies using high-throughput array platforms, we identified 13 candidate sncRNA biomarkers that are differentially regulated in PBMC of mTBI compared to non-TBI control cases. In follow-up Biomarker Validation studies using an independent cohort of 58 veteran cases, we confirmed that 4 small nucleolar RNAs (snoRNAs), ACA48, U35, U55, and U83A, are significantly down-regulated in PBMC from veterans with mTBI and PTSD compared to non-TBI, control subjects with PTSD only. The snoRNA biomarkers are able to dissect subjects with comorbid mTBI and PTSD from PTSD subjects without mTBI with 100% sensitivity, 81% accuracy, and 72% specificity. No significant differential expression of snoRNA biomarkers was found in mTBI subjects without comorbid PTSD. With the exception of U55, we also found no significant differential expression of snoRNA biomarkers in subjects with PTSD. Additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD. Outcomes from our studies suggest that biological interactions between TBI and PTSD may contribute to the clinical features of mTBI with comorbid PTSD. Thus, ongoing and future investigations on mTBI mechanisms or TBI biomarkers should carefully consider their interactions with PTSD. Future studies clarifying the bioactivities of ACA48, U55, and U83A will provide insights on pathogenic mechanisms contributing to long-term clinical complications in veterans with comorbid TBI/PTSD.

15. SUBJECT TERMS
Operation Iraqi Freedom (OIF);Operation Enduring Freedom (OEF); Traumatic Brain Injury (TBI); Post-traumatic stress disorder (PTSD)

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1.INTRODUCTION

Our overall goal is to identify, characterize, and validate clinically accessible molecular biomarkers of mild traumatic brain injury (mTBI) from peripheral blood mononuclear cells (PBMC), which could be used to help identify returning Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) Veterans who are suffering from the effects of mTBI. The study was conducted in two stages. We first conducted a Biomarker Discovery study in which we used a high throughput Array Chip platform to quantify PBMC expression of 1,500 small non-coding (snc) RNAs and identify candidate mTBI biomarkers that are differentially regulated in veterans with mTBI compared to veterans without mTBI. We then conduct a Biomarker Validation study in which we used quantitative real-time PCR (qPCR) assays to validate the ability of the candidate sncRNA mTBI biomarkers to diagnose mTBI in an independent OIF/OEF veteran study cohort in the presence or absence of post-traumatic stress disorder (PTSD) comorbidity. Outcomes from our studies suggest that biological interactions between TBI and PTSD may contribute to the clinical features of mTBI with comorbid PTSD. Thus, ongoing and future investigations on mTBI mechanisms or TBI biomarkers should carefully consider their interactions with PTSD.

2.KEYWORDS

Anxiety
biomarker
chronic social defeat
comorbidity
depression
inflammatory
microRNA
mild traumatic brain injury
Operation Enduring Freedom
Operation Iraqi Freedom
oxidative stress and
peripheral blood mononuclear cells
post-traumatic stress disorder
small non-coding RNA
small nucleolar RNA
veterans

3. OVERALL PROJECT SUMMARY

3A. Our Statement of Work (revised) defined three Project Tasks as follow

Task 1: Our original plan was to recruit a large cohort of healthy and mild traumatic brain injury (mTBI) cases among veterans who were deployed to Afghanistan (OEF) and Iraq (OIF) and who were previously seen by the
The War Related Illness and Injury Study Center (WRIISC) clinical team at the Department of Veterans Affairs, New Jersey Health Care System (DVANJHCS), East Orange, NJ., for comprehensive health evaluations. Our original goal was to recruit a total of 80 OEF/OIF veterans: 40 who were deemed positive for mTBI and 40 who were not, and to prepare and bank peripheral blood mononuclear cell (PBMC) specimens from freshly collected blood samples from all recruited cases at recruitment (baseline) for subsequent "Biomarker Discovery" studies. For this "retrospective" part of the study, our plan was to only contact those veterans who have previously consented to being contacted for future research studies at the WRIISC (months 6-24) The first 6 months of the project period has been set aside for second-tier regulatory review by the Army's Office of Research Protections.

A. Veterans previously identified as either positive or negative for mTBI, based on operationally defined clinical criteria for mild TBI, will be invited for a study visit to the WRIISC for a blood draw(months 6-24).
B. At the time of their visit to the WRIISC, the Veterans will be administered a standardized, repeatable neuropsychological screening exam of no longer than 60 minutes duration (months 6-24).

Our plan was to use banked PBMC specimens from this "Biomarker Discovery" cohort in small noncoding (snc) RNA expression profile analysis studies (see Tasks 3, below) to identify candidate mTBI biomfarker sncRNA species that are differentially regulated in mTBI vs. non-TBI control cases.

After completion of an interim Biomarker Discovery studies using a cohort of 17 "Biomarker Discovery" cases (8 TBI and 9 non-TBI cases), we identified 18 candidate sncRNA biomarkers that are significantly differentially-regulated in TBI vs. non-TBI control cases. In follow-up studies using independent quantitative real time polymerase chain reaction (qPCR) protocols, we confirmed that 13 of the 18 candidate sncRNA biomarkers are, indeed differentially regulated in PBMCs of TBI compared to non-TBI control veteran cases in the Biomarker Discovery study cohort. Based on this, we proposed to focus all our efforts to validate the accuracy, sensitivity, and specificity of individual or panels of the 13 qPCR-confirmed biomarkers in a new cohort of TBI and non-TBI cases as we originally proposed. Continuation of Validation studies will be discussed in more details in Tasks 2 and 3.

Task 2: Concurrent with Task 1 that was designed to collect samples for "Biomarker Discovery" studies, our Task 2 studies were designed to recruit 200 OEF/OIF veterans at the WRIISC for "Biomarker Validation" studies and to collect and bank PBMC specimens from all 200 cases. We anticipate 20% (or 40 cases) of the samples would be subsequently identified as mTBI cases (months 12-30).

A. Going forward, all OEF/OIF veterans that will be seen at the WRIISC for clinical evaluations will be asked to participate in the study at the time of their visit or to return within seven (7) business days from the date of their clinical evaluation to provide consent for a blood draw, regardless of TBI status (months 12-30).
B. Those who agree to participate in the study will NOT be administered a standardized, repeatable neuropsychological screening exam because that is already part of their comprehensive health examination which would have been completed on the same day as the consent process and blood sampling

Our original plan was to analyze samples collected from the "Biomarker Validation" cohort in Task 2 using independent Q-PCR protocols (see Task 3, below) to confirm the efficacy of individual or panels of candidate mTBI biomarkers (identified in Task 1) to correctly identify mTBI cases.

Based on the identification of 13 candidate small RNA TBI biomarkers from our Biomarker Discovery studies, our Task 2 "Biomarker Validation" studies are now focused on validating the accuracy, sensitivity, and specificity of individual (or panels of) biomarkers in a new cohort of TBI and non-TBI cases as we original proposed. We currently have 82 cases (16 TBI and 66 non-TBI cases) that are available for Biomarker Validation studies. The number of TBI cases we currently have is not sufficient for our proposed Biomarker Validation studies. We anticipate we will need a total of 23 TBI and 23 age- gender-matched non-TBI controls for our Biomarker Validation studies. As summarized in Table I (below), we will need to recruit an additional 7 TBI cases for our Biomarker Validation studies. Based on our estimation that 25% of the recruits will be identified as TBI, we anticipate we will need to recruit a total of 28 new cases (comprised of 7 TBI and 21 non TBI cases) for the Biomarker Validation Study Cohort before we have sufficient number of TBI cases for Biomarker Validation studies (see Table I) as we proposed for Task 2.

	TBI	Non-TBI control
Cases currently available for Biomarker Discovery studies	16	66
28 new cases needed to be recruited for Biomarker Validation studies	7	21
Total number of cases that will be available for Biomarker Validation studies	23	87
Total number of cases that will be use for Biomarker Validation studies	23	23

Table I: Summation of case for our Biomarker Validation studies. As indicated, we currently have 16 TBI and 66 non-TBI cases for our Biomarker Validation studies. We need to recruit 28 new cases, which we anticipate will be comprised of 7 TBI and 21 non-TBI control cases. Thus, we will have 23 TBI and 87 non-TBI cases from which we will use 23 TBI and 23 age-, gender-matched non-TBI control cases for our final Biomarker Validation studies

Task 3: Our Task 3 studies are designed to have blood samples collected from Tasks 1 and 2 be processed at the Bronx VA to identify candidate miRNA fingerprints that could be used to distinguish veterans having mild TBI from those without (months 12-36).

A. Dr. Giulio Pasinetti from the Bronx VA will be provided with the de-identified blood samples collected from the "Biomarker Discovery" and the "Biomarker Validation" cohort for microRNA and real-time PCR analysis, respectively. Dr Pasinetti and his team will be blind to group designation (months 6-36).
B. We will analyze microRNA expression profiles of banked blood specimens from 40 mTBI and 40 healthy control cases in the "Biomarker Discovery" cohort to identify specific candidate mTBI microRNA biomarker species that are differentially expressed in the banked PBMC specimens of mTBI vs. healthy control cases in the "Biomarker Discovery" cohort (months 12-24).
C. We will subsequently use independent, quantitative, real-time PCR assays in the analysis of banked blood specimens from the "Biomarker Validation" cohorts to test the efficacy of individual or panels of candidate mTBI miRNA biomarkers to correctly identify mTBI cases (months 24-36).

3B. Study sites

Our study was conducted in collaboration by two sites identified below. The WRIISC site was responsible for recruitment of veteran subject and collection and banking of PBMC specimens from all recruits. Banked PBMC was sent to The Bronx Veterans Medical Research Foundation site analysis of RNA biomarker studies.

Site 1: Bronx Veterans Medical Research Foundation, Inc.
130 West Kingsbridge Road
Bronx, NY 10468

Site 2: War Related Illness and Injury Study Center (WRIISC)
Department of Veterans Affairs, New Jersey Health Care System (DVA NJHCS)
385 Tremont Avenue
East Orange, NJ 07018

3C. Key Methodologies used

Participant recruitment: OIF and OEF veteran cases with our without a history of mTBI were recruited by The War Related Illness and Injury Study Center (WRIISC), Department of Veterans Affairs, New Jersey Health Care System (DVANJHCS), East Orange, NJ. Male and female participants were included if they were between 18-75 years of age and had completed a clinical evaluation at the New Jersey WRIISC. Cases with intercurrent infections or inflammatory-related conditions were excluded. Participants were classified as having a history of mTBI if they met at least one of 4 criteria on the veteran traumatic brain injury screening tool (VAT-BIST) (Donnelly et al., 2011), and had a score at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS) (Randolph, 1998). Classification criteria for control cases included a negative VAT-BIST score and a RBANS score less than one standard deviation below the norm. Human participants were recruited by the WRIISC following voluntary written informed consent.

PBMC isolation: Blood specimens from human subjects were collected by venipuncture and drawn into BD Vacutainer CPT Cell Preparation Tubes (Becton, Dickinson and Company). PBMCs were isolated from freshly collected blood specimens according to the manufacturer's instructions, and were stored at -80 ^{o}C until use.

Biomarker studies: Small noncoding RNA expression profile analysis was conducted using techniques we developed for gathering of feasibility evidence in support of our original application. We first extracted total RNA from banked PBMC using Triazol (Invitrogen) reagent following standard methodologies. Thereafter, sncRNA species in isolated RNA specimens was end-labelled with Cy3 followed by hybridization of labeled miRNA to the *Agilent* miRNA platform. SncRNA labeling reactions was done commercially, carried out by Cogenic, Inc., using *Agilent's* miRNA system according to the manufacturer's protocol using the Exiqon mirCURY LNA Array labeling kit. Approximately 100 ng of total RNA was used per reaction. SncRNA hybridizations was conducted on a Tecan 4800HS hybridization station, using *Agilent's* protocol with channel definitions as follows: channel 1 - 2xSSC, 0.2 percent SDS; channel 2 - 2xSSC; and channel 3 - 0.2xSSC. Slides were scanned using the Molecular Dynamics GenePix 4000B scanner. Images were analyzed and data extracted using the GenePix Pro version 6.0 software package. Quality assurance measures included routine inclusions of 'doped-in' control RNA. As we have done in our feasibility studies, we used t-statistic, followed by applications of false-discovery rate procedures for multiple test correction to identify candidate differentially regulated sncRNA species between the mTBI and healthy cases in the “Biomarker Discovery” cohort.

For quantitative real-time RT-PCR studies, we used Taqman microRNA Reverse Transcription Kits specific for individual targeted sncRNA, in conjunction with Taqman MicroRNA Assays, and Taqman 2x Universal PCR Master Mix (No AmpErase LING) (all from Applied Biosystems, USA) with 10 ng of total RNA, as we have described in our original application. The U43 small nucleolar RNA primer (RNU43; Applied Biosystems) ws used as a reference. Reverse transcription reactions were first incubated on a PTC-200 thermal cycler (MJ Research) for 30 min. at 16 °C, 30 min. at 42 °C, 5 min. at 85 °C and then held at 4 °C. Real-time PCR reactions were performed in four replicates on an ABI Prism 7900HT (Applied Biosystems) at 95 °C for 10 min., followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min., according to the manufacturer's instructions. SncRNA fold changes between groups are then calculated using the ACt method. Statistical analysis of individual sncRNA expression between the two cohorts will be assessed using t-statistics.

Description of any changes to originally proposed methods:

Following our Biomarker Discovery studies, we have unused samples from 29 cases that were originally collected for Biomarker Discovery studies. In 2010, we obtained NJ Healthcare System IRB approval to reclassify these unused cases for Biomarker Validation studies. We attached a copy of a notification from the NJ Healthcare System IRB, granting approval for reclassifying the 29 unused cases

3D. Summary of Results, Progress and Accomplishments

3D1. Tasks 1 and 3 Results, Progress and Accomplishments:

We conducted an initial Biomarker Discovery study using a high throughput Array Chip platform. We identified candidate sncRNA biomarker, including microRNA (miRNA) and small nucleolar RNA (snoRNA), whose expression in circulating blood monocytes (PBMC) is capable of dissecting OEF/OIF veterans with TBI from non-TBI control OEF/OIF veterans. Our Biomarker Discovery study was conducted using 9 mild TBI cases and 9 control non-TBI cases matching TBI cases for demographic and co-morbidities. Demographic information for individual mTBI and non-mTBI cases is presented in Table II (below).

case	TBI / Ctl	Age	Gender	Ethnicity	Interval (yrs) since last deployment	Education (yrs)	CI-morbidity (PTSD)
31529	TBI	38	Male	Black, non- Hispanic	3	14	Yes
33297	TBI	41	Male	Native American	4.3	12	Yes
33825	TBI	31	Male	Black, non- Hispanic	3.4	16	Yes
33828	TBI	42	Male	Black, non- Hispanic	0.7	14	Yes
33888	TBI	27	Female	White Hispanic	4.5	14	Yes
33931	TBI	23	Male	White Hispanic	1.2	12	Yes
33947	TBI	25	Female	Black, non- Hispanic	2.8	13	No
33881	TBI	32	Male	White Hispanic	10.2	13	Yes
33811	TBI	27	Male	Black, non- Hispanic	4.8	12	Yes
31705	Non TBI Ctl	27	Male	Black, non- Hispanic	3.4	12	No
33565	Non TBI Ctl	25	Male	White Hispanic	1.1	12	Yes
33578	Non TBI Ctl	30	Female	White Hispanic	4.3	16	Yes
33596	Non TBI Ctl	35	Male	White Hispanic	3.8	16	Yes
33598	Non TBI Ctl	49	Male	White Hispanic	0.7	9	Yes
33821	Non TBI Ctl	26	Female	White Hispanic	0.2	16	No
33834	Non TBI Ctl	24	Male	White Hispanic	2.8	12	Yes
33913	Non TBI Ctl	22	Female	Black, non- Hispanic	6.5	12	Yes
33930	Non TBI Ctl	30	Male	Black, non- Hispanic	1	12	Yes

Table II. Demographic characteristics of mTBI and non-mTBI control cases we used in our interim Biomarker Discovery study*. Mild TBI classification is based on positive endorsement of the VA traumatic brain injury screen (VAT-BIS) and a score of at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS). Non-mTBI case classification is based on negative endorsement of VAT-BIS and a RBANS score of less than one standard deviation below the norm. PTSD diagnosis is based on a score of 50 or more on the PTSD Checklist – Civilian Version. Average age: mTBI group, 31.6±7.0 yrs; non-mTBI group, 29.8±8.2 yrs. Interval between their last deployment and recruitment into this study: mTBI group, 3.9±2.7 yrs; non mTBI group 2.6±2.1 yrs. Average duration of education: mTBI group, 13.3±1.3; non-mTBI group, 13.0±2.4 yrs. Percentage of males: mTBI group, 78%; non-mTBI group, 67%. Percent of cases with co-morbid PTSD: mTBI group, 89%; non-mTBI group, 78%. We note that the majority of Veterans with mTBI in our biomarker study have co-morbid PTSD and that our non-mTBI cases are selected to match for PTSD diagnosis.*

Identification of candidate RNA mTBI biomarkers using a high throughput Array platform

High through-put study was conducted using the Affymetric Human gene 1.0 ST Array chip that is designed to analyze the expression of 1500 small RNAs such as microRNA as well as small nucleolar RNA, small cytoplasmic RNA and ribosomal RNA. We detected from our human PBMC specimens a total of 428 small RNA species, including 190 microRNAs, 220 small nucleolar RNAs, 8 small cytoplasmic RNAs and 10 ribosomal RNAs. Based on principal components analysis of all signals detected, we observed that one TBI

case (case #33811) might be an outlier (data not shown). Due to this and the fact that the quality of RNA extracted from this case is poor, we decided to remove case #33811 from subsequent statistical analysis.

Statistical analysis to identify candidate TBI biomarkers were conducted using 8 TBI (minus case #33811) and 9 control non-TBI cases. Two criteria were used to identify candidate small RNA biomarkers for TBI: 1) group changes (TBI vs. control groups) must be associated with a magnitude of $\geq$ 1.5-folds, and 2) group changes must be statistically significant with $p < 0.05$, based on t-test analysis followed by the application false discovery rate corrections for multi-sampling errors. We identified 18 candidate small RNA biomarkers meeting both criteria that are significantly differentially-regulated in TBI vs. control cases: 4 microRNAs, 13 snoRNAs and 1 small cytoplasmic RNA. Using the 18 candidate small RNA biomarker in an unsupervised clustering analysis, we were able to correctly segregate all 17 TBI and control cases analyzed in this interim study (Fig. 1, below).

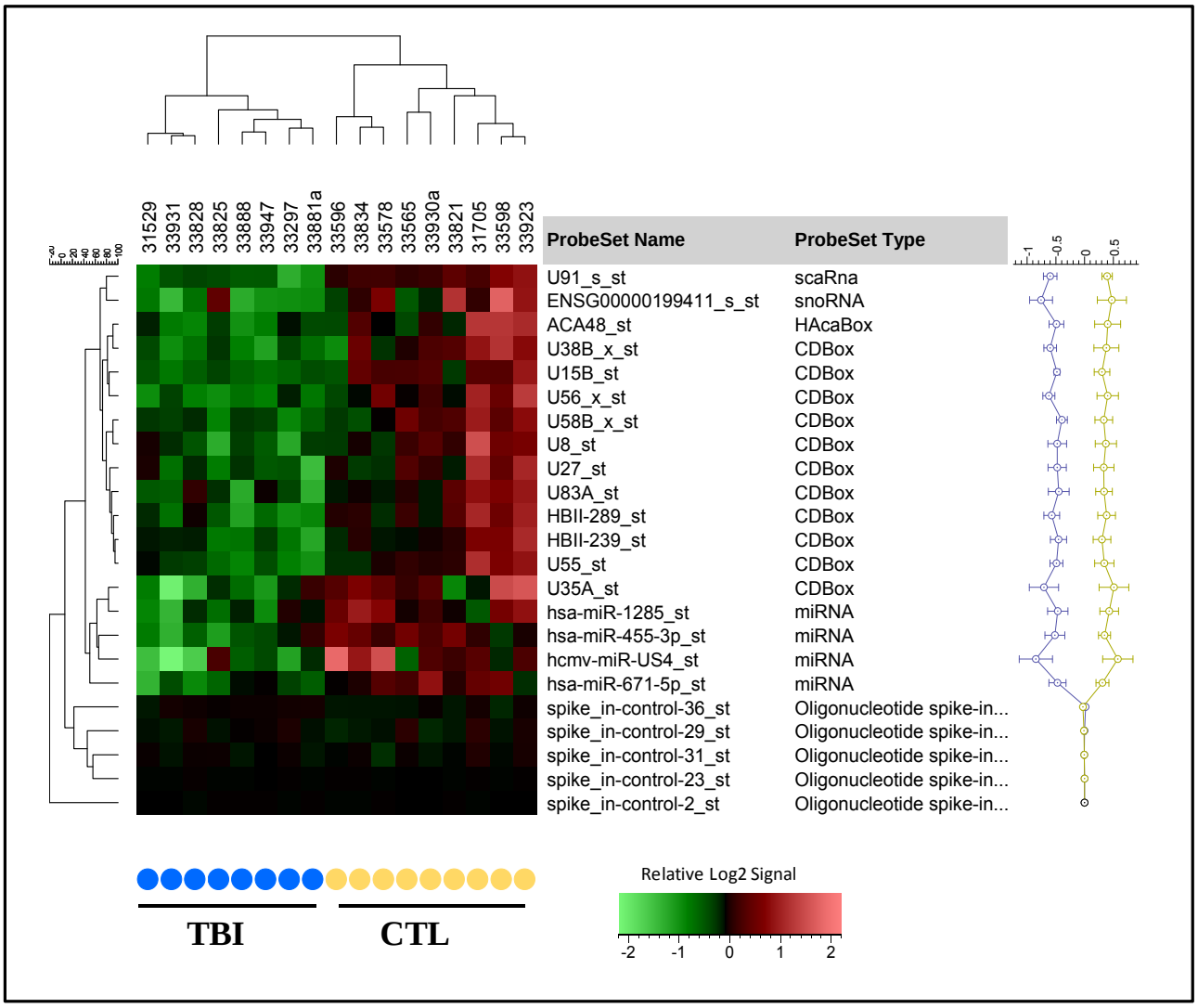


Figure 1: Unsupervised clustering analysis of 18 candidate small RNA TBI biomarker species. *The 18 differentially-regulated small RNAs identified from interim analysis of 8 TBI and 9 control cases are subjected to unsupervised hierarchical clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are present as a heat map (left panel), which showed that a panel of 18 small RNA biomarker species is able to correctly segregate TBI from control cases. Names for each of the small RNA biomarker species are identified under "Probe Set Name". Small RNA classes (and subclasses) these 18 differentially-regulated TBI biomarkers belonging to are shown under "Probe Set Type". Vertical dendrogram (right panel) presents average (+/- SD) signal detections from TBI vs. control groups for each of the 18 candidate small RNA biomarkers and confirmed divergent regulations of the biomarkers in PBMC of TBI vs. control groups. Differential regulations of the 18 candidate biomarkers likely reflect true biological effects and not systematic experimental artifact (s) since no group differences are observed for the detection of spike-in control oligonucleotides in all 17 OIF/OEF veteran cases analyzed (see heat map and vertical dendrogram).*

Abbreviations: miRNA, microRNA; snoRNA, small nucleolar RNA; C/D Box, the C/D box subclass of small nucleolar RNA; HAc Box, the HAc Box subclass of small nucleolar RNA; scaRNA, small cytoplasmic RNA.

We noted that our interim analysis suggests that several classes of small RNA biomarker species might be differentially regulated in PBMC from TBI vs. control OEF/OIF veterans. MicroRNAs are known to modulate mRNA stability/translation, while small nucleolar RNAs are known to help guide RNA modifications and small cytoplasmic RNAs are known to interact and guide intracellular transports of select proteins. Thus, further understandings on specific RNA/protein interacting with these candidate small RNA biomarkers will likely provide insights on critical cellular and/or physiological mechanisms underlying TBI clinical complications.

Independent qPCR validation of candidate small non-coding RNA biomarkers

In follow-up studies using independent quantitative real time polymerase chain reaction (qPCR) protocols, we confirmed that 13 of the 18 candidate small RNA biomarkers are, indeed, differentially regulated in the PBMC of TBI compared to non-TBI veteran cases (Fig. 2, below). The 13 confirmed small RNA biomarkers include 12 snoRNA (ACA48, ENSG199411, HBII-239, HBII-289, U15B, U27, U35A, U55, U56, U58B, U83A, U91) and 1 miRNA (Has-miR-671-5p) (Fig. 2). Each of the 13 confirmed small RNA biomarkers are found in significantly lower levels in PBMC specimens from TBI, compared to non-TBI control veteran cases (Fig. 2).

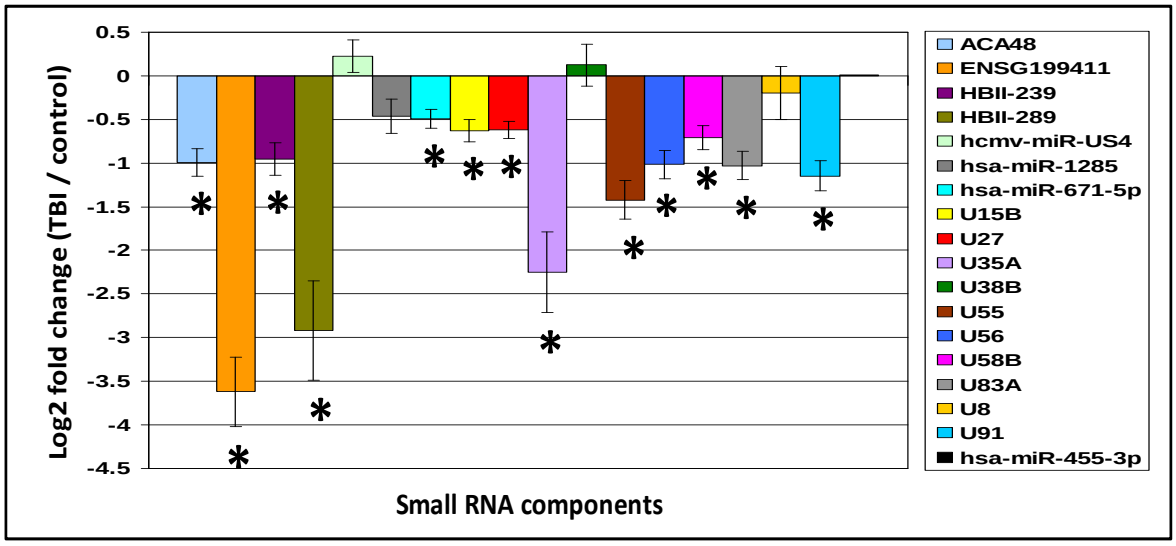


Figure 2: Q-PCR assays confirmed 13 small RNA TBI biomarkers are differentially regulated in PBMC of TBI relative to non-TBI control cases. *PBMC contents for each of the18 candidate small RNA biomarkers were quantitatively assessed using independent Q-PCR assays. The same 9 TBI and 9 control non-TBI cases we used in our initial high-throughput biomarker discovery assay were assessed in this Q-PCR Biomarker confirmation studies. Bar graphs represent mean small RNA biomarker contents in the TBI group relative to the non-TBI control group; error bars represent standard errors. * False discovery rate-corrected P-value < 0.05. Q-PCR confirmed 13 small RNA biomarker species are significantly down-regulated in PBMC of TBI compared to non-TBI control veteran cases.*

PTSD is commonly co-morbid with TBI in OEF/OIF veterans. Consistent with this, the majority of TBI cases in our biomarker study are co-morbid with PTSD (Table II). Therefore, our non-TBI control cases were selected to match for PTSD diagnosis. Thus, our identified panel of 13 small RNA biomarkers likely represents biological indices selective for TBI. Moreover, TBI cases in our biomarker discovery studies were recruited after an average interval of 3.9 years following their last deployment (deployment-to-recruitment interval: ranging from 0.7 to 10.2 years, with a median interval of 3.4 years). The changes we observed in the regulation of these small RNA TBI biomarkers are not acute TBI responses, but likely represent long-term pathophysiological consequences subsequent to TBI.

3D2. Tasks 2 and 3 Results, Progress and Accomplishments

Based on identification of the 13 candidate sncRNA biomarkers from our Biomarker Discovery cohort for which the majority of mTBI and control subjects had comorbid PTSD, we conducted a Biomarker Validation

study to explore, in a new veteran cohort, the ability of these candidate biomarkers to distinguish mTBI in the presence or absence of PTSD comorbidity. The Biomarker Validation cohort was comprised of 13 mTBI cases and 45 non-TBI cases. The proportion of veterans (22%) classified with mTBI in our recruited cohort was consistent with, and even slightly above, previous prevalence estimates of 12% reported in a cross-sectional survey of 2,235 active duty, guard, and reserve OEF/OIF veterans (Schneiderman et al., 2008). Demographic information for the study cohort was shown in Table III (below). Notably, our study cohort contained mTBI and non-TBI cases with or without co-morbid PTSD. This presents the opportunity to explore the ability of our previously identified candidate sncRNA biomarkers to distinguish mTBI in the presence or absence of PTSD comorbidity

***PBMC expression of the candidate biomarkers in mTBI and control non-TBI cases comorbid with PTSD*:**
Our Biomarker Validation study cohort contained 6 mTBI/PTSD and 11 non-TBI/ PTSD cases (Table III). There were no significant differences between the mTBI/PTSD and non-TBI/PTSD groups with respect to group average values for age (31.5$\pm$10.7 and 33.1$\pm$11.08 years, respectively; p=0.67), post-deployment interval (5.3±2.5 and 3.6±2.5 years, respectively; p=0.20), or years of education (14.7±2.5 and 13.3±2.6 years; p=0.18). The proportion of males in the mTBI and the non-mTBI groups were 83% and 82%, respectively.

We quantified PBMC contents of individual candidate small noncoding RNA mTBI biomarkers in each of the cases, using qPCR. Consistent with observations from our initial exploratory biomarker discovery studies (Figure 2, above), we that found that 4 of the small noncoding RNA biomarkers, ACA48, U35A, U55, and U83A, are significantly down-regulated in mTBI/PTSD cases compared to non-TBI/PTSD cases (Fig. 3B, below). Our observations validate, for the first time, ACA48, U35A, U55, and U83A in PBMC as biomarkers of mTBI in the context of PTSD comorbidity. Moreover, using unsupervised hierarchical cluster analysis, we found that a combination of ACA48, U35A, U55, and U83A was able to correctly distinguish mTBI/PTSD cases from control non-TBI/PTSD cases with 82% accuracy, 100% sensitivity, and 72% specificity (Fig. 3C, D). The sensitivity and specificity of using the combined biomarker for correctly segregating mTBI/PTSD vs. non-TBI/PTSD cases in our present study cohort was confirmed using the receiver operating characteristic (ROC) analysis as an independent assessment.

We note that all 4 validated biomarkers of mTBI/PTSD comorbidity are members of the small nucleolar RNA (snoRNA) class that are known for their activities in modulating RNA splicing, stability, and/or translation (Lui et al, 2013, Kishore et al., 2006, Sridhar et al., 2008). Potential relevance of the 4 snoRNA biomarkers to TBI/PTSD pathophysiology will be discussed in more details in the *Discussion* section (section 3D), below.

Case #	mTBI/non-TBI	age	sex	Ethnicity	Interval (yrs) since last deployment	Education (yrs)	PTSD Comorbidity
33361	mTBI	26	F	Wh/Hisp	3.2	13	Yes
34084	mTBI	32	M	Bl/Afr Am/nHisp	3.5	13	Yes
34119	mTBI	29	M	Wh/nHisp	8.1	18	Yes
34124	mTBI	47	M	Wh/nHisp	2.6	18	Yes
34193	mTBI	51	M	Wh/nHisp	6.7	13	Yes
34260	mTBI	28	M	Bl/Afr Am/nHisp	7.9	13	Yes
33478	mTBI	30	M	Wh/nHisp	6.4	18	No
33570	mTBI	23	M	Wh/Hisp	2.8	13	No
34055	mTBI	28	M	Wh/Hisp	3.2	14	No
34128	mTBI	24	M	Wh/nHisp	1.5	12	No
34138	mTBI	26	M	Wh/nHisp	2.6	12	No
34254	mTBI	35	M	Wh/nHisp	7.2	15	No
34261	mTBI	26	M	Wh/nHisp	3.7	13	No
33759	non-TBI	28	M	Wh/Hisp	0.4	12	Yes
33899	non-TBI	60	M	Wh/nHisp	not deployed	14	Yes
33913	non-TBI	22	F	Bl/Afr Am/nHisp	1.0	12	Yes
33944	non-TBI	30	M	Wh/nHisp	8.4	14	Yes
33848	non-TBI	25	M	Bl/Afr Am/nHisp	4.3	13	Yes
33977	non-TBI	42	M	Bl/Afr Am/nHisp	2.3	14	Yes
34060	non-TBI	27	F	Wh/nHisp	3.9	17	Yes
34070	non-TBI	24	M	Wh/Hisp	1.2	12	Yes
34079	non-TBI	37	M	Wh/nHisp	4.3	14	Yes
34087	non-TBI	40	M	Wh/Hisp	4.4	12	Yes
34160	non-TBI	29	M	Wh/nHisp	6.0	12	Yes
30597	non-TBI	40	M	Wh/nHisp	4.4	18	No
31429	non-TBI	39	M	Bl/Afr Am/nHisp	0.9	14	No
31824	non-TBI	43	M	Bl/Afr Am/nHisp	6.5	13	No
33673	non-TBI	25	M	Wh/Hisp	2.8	14	No
33676	non-TBI	32	M	Wh/Hisp	1.0	16	No
33699	non-TBI	42	M	Bl/Afr Am/nHisp	0.7	16	No
33765	non-TBI	26	F	Wh/Hisp	0.9	16	No
33786	non-TBI	52	M	Bl/Afr Am/nHisp	0.7	14	No
33819	non-TBI	24	M	Wh/Hisp	1.9	12	No
33832	non-TBI	43	M	Wh/nHisp	4.4	12	No
33858	non-TBI	29	M	Bl/Afr Am/nHisp	6.5	18	No
33863	non-TBI	41	M	Wh/nHisp	0.8	12	No
33864	non-TBI	23	M	Wh/Hisp	1.8	14	No
33877	non-TBI	59	M	Wh/nHisp	0.7	16	No
33904	non-TBI	22	M	Asian	0.6	12	No
33905	non-TBI	38	M	Wh/nHisp	1.1	18	No
33911	non-TBI	29	M	Bl/Afr Am/nHisp	7.7	16	No
33915	non-TBI	25	F	Bl/Afr Am/nHisp	4.2	14	No
33918	non-TBI	33	M	Wh/Hisp	1.7	12	No
33958	non-TBI	23	M	Wh/Hisp	1.2	12	No
33992	non-TBI	28	M	Wh/nHisp	4.7	13	No
34026	non-TBI	34	M	Asian	1.3	13	No
34047	non-TBI	45	F	Bl/Afr Am/nHisp	4.9	15	No
34076	non-TBI	40	M	Bl/Afr Am/nHisp	0.7	12	No
34077	non-TBI	25	M	Wh/Hisp	1.2	13	No
34093	non-TBI	47	M	Wh/nHisp	1.9	16	No
34094	non-TBI	42	M	Wh/nHisp	3.8	17	No
34117	non-TBI	42	M	Wh/nHisp	0.4	20	No
34120	non-TBI	54	M	Wh/nHisp	5.2	16	No
34126	non-TBI	34	M	Wh/Hisp	6.8	13	No
34142	non-TBI	46	M	Wh/Hisp	1.7	14	No
34192	non-TBI	24	M	Wh/Hisp	1.6	12	No
34247	non-TBI	26	F	Wh/Hisp	1.0	13	No

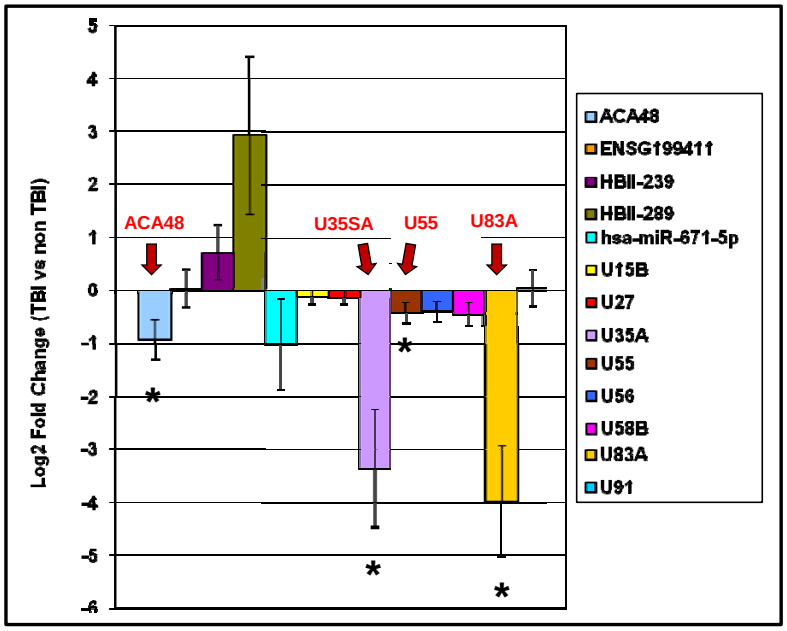
Table III: Demographic characteristics of the Biomarker Validation study cohort. *Information is presented for the 58 cases recruited for this study. Cases are subgrouped by characterization of mTBI with comorbid PTSD, non-TBI with PTSD, non-TBI without PTSD, and non-TBI without PTSD. Abbreviations; Wh, white; Hisp, hispanic; nHisp, non-hispanic; Bl, black; Afr Am, African American. There were no significant differences between the mTBI and non-TBI groups with respect to group average values for age*

(31.2±8.6 and 34.8±10.3 years, respectively; p=0.25) or years of education (14.2±2.3 and 14.1±2.1 years, respectively; p=0.83). The mTBI group had a significantly longer time interval between their last deployment and their recruitment into this study (4.6±2.3 for the mTBI group and 2.8±2.3 years for the non-TBI group, p = 0.02). The proportion of males in the mTBI and the non-mTBI groups were 92% and 87%, respectively.

A

Candidate small RNA Biomarker species	mTBI/PTSD vs. non-TBI/PTSD
ENSG00000199411	0.47
hsa-miR-671-5p	0.50
ACA48	0.05
HBII-239	0.35
HBII-289	0.19
U15B	0.63
U27	0.72
U35A	0.05
U55	0.05
U56	0.14
U58B	0.12
U83A	0.007
U91	0.91

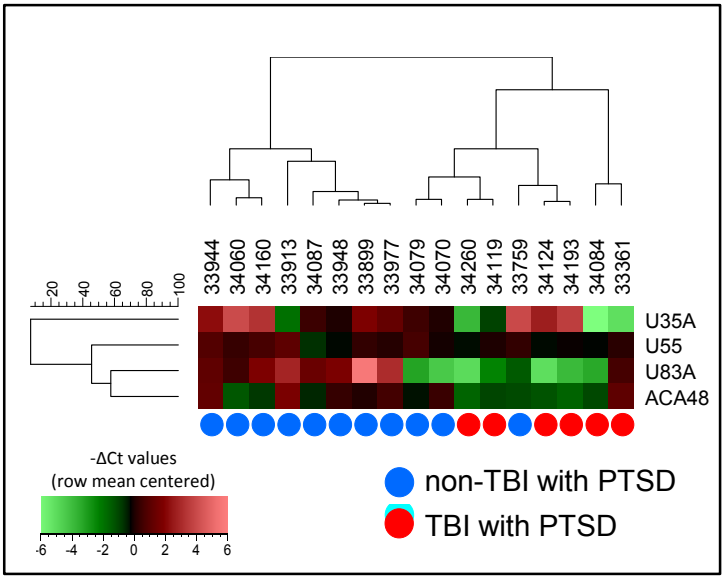
B



C

Biomarker combinations	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	6	3	8	0	1.00	0.51-1.00	0.72	0.39-0.93	0.82
ACA48/U35A/U83A	6	2	9	0	1.00	0.51-1.00	0.81	0.47-0.97	0.88
U35A/U55/U83A	6	2	9	0	1.00	0.51-1.00	0.81	0.47-0.97	0.88
ACA48/U55/U83A	5	3	8	1	0.83	0.36-0.99	0.72	0.39-0.93	0.76
ACA48/U35A/U55	4	3	8	2	0.66	0.24-0.94	0.72	0.39-0.93	0.71
U35A/U83A	6	2	9	0	1.00	0.51-1.00	0.81	0.47-0.97	0.88

D



E

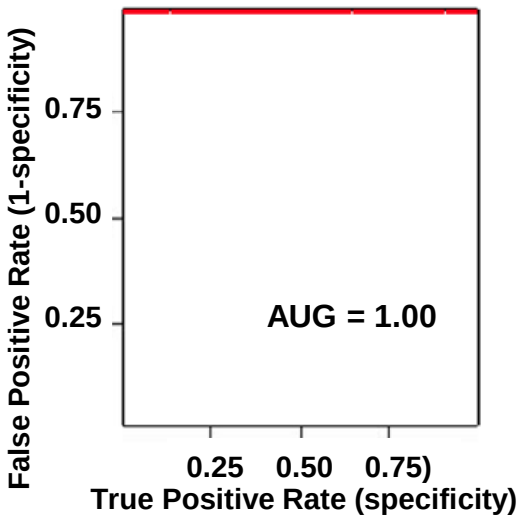
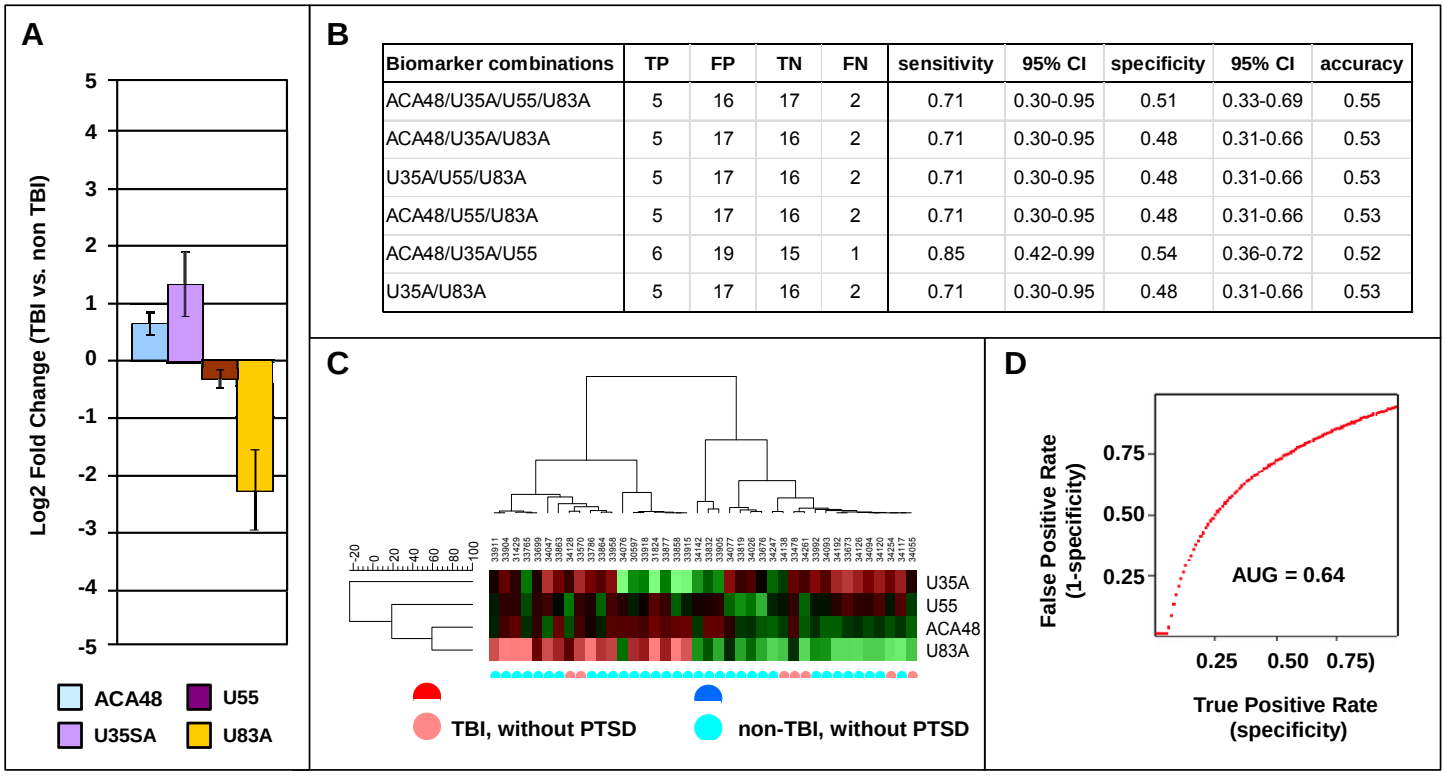


Figure 3: Candidate mTBI small noncoding RNA biomarker expression in TBI vs non-TBI cases in the context of PTSD comorbidity. *(A, B) Contents of* candidate small noncoding RNA biomarkers *in PBMC from mTBI/non-PTSD and non-TBI/non-PTSD cases were assessed by qPCR. A) t-test p-value comparisons between mTBI/PTSD vs. non-TBI/PTSD cases. Red letters*

highlight candidate small noncoding RNA mTBI biomarkers with significant differential regulation in PBMC specimens of mTBI/PTSD compared to non-TBI/PTSD cases. B) Bar graphs show mean±SEM fold change (in Log2 values) of candidate mTBI biomarkers in mTBI/PTSD vs. control non-TBI/PTSD cases. Values <0 or >0 indicate, respectively, down-regulation or up-regulation in mTBI/PTSD compared to non-TBI/PTSD cases. Arrows point to the 4 small noncoding RNAs that were significantly down-regulated (p<0.05) in mTBI/PTSD vs. control non-TBI/PTSD cases. (C, D, E)* The combination of a 4-biomarker panel, *ACA48, U35A, U55, and U83A*, provides a sensitive and specific criterion for differentiating mTBI/PTSD from non-TBI/PTSD cases. *Unsupervised hierarchical clustering of mTBI/PTSD and non-TBI/PTSD cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples into one of two major clusters. One of the major clusters contained a majority of the mTBI samples and was designated as the mTBI cluster, while the other major cluster was designated as the non-TBI cluster.* A) *Summation table of the unsupervised hierarchical clustering analyses using combinations of the 4 biomarkers, ACA48, U35A, U55, and U83A, to correctly identify mTBI/PTSD vs. non-TBI/PTSD cases. Accuracy represents the percentage of all mTBI/PTSD and non-mTBI/PTSD cases that were correctly diagnosed by the test, calculated as the number of correctly identified mTBI/PTSD and non-TBI/PTSD cases divided by the total number of cases analyzed. Sensitivity (true positive [TP]/[TP + false negative (FN)]) is the probability that a case predicted to have mTBI actually had it, whereas specificity (true negative [TN]/[false positive (FP) + TN]) measures the probability that a case predicted not to have mTBI did, in fact, not have it. (B) Heat map of an* unsupervised hierarchical clustering analysis using the combined 4 small noncoding RNA biomarkers (*ACA48, U35A, U55, and U83A) showing segregation of mTBI/PTSD and non-TBI/PTSD cases. C) Receiver Operating Characteristic (ROC) analysis using the combined 4 biomarkers. ROC curve plotting the percentage of correctly identified mTBI/PTSD cases (true positive; specificity) as a function of the percentage of non-TBI/PTSD cases incorrectly identified as mTBI/PTSD cases (false positive; 1-specificity). The calculated area under the curve (AUC) is 1.0. In general, an AUC of 1 indicates the capability of a test to perfectly segregate two populations, and an AUC of 0.5 indicates that the test cannot segregate two populations beyond chance. This evidence supports the hypothesis that the combined 4 small noncoding RNA biomarker is a vigorous and sensitive test to distinguish mTBI/PTSD from non-mTBI/PTSD cases.*

PBMC expression of ACA48, U35A, U55, and U83A in mTBI and control non-TBI cases without PTSD: We continued to explore the regulation of the 4 snoRNA biomarkers in mTBI and non-TBI cases in the absence of PTSD. Our study cohort of 58 veteran cases contained 7 mTBI cases without PTSD and 34 non-TBI cases without PTSD (herein referred to as mTBI/non-PTSD and non-TBI/non-PTSD cases, respectively). Demographic information for the mTBI/non-PTSD and non-TBI/non-PTSD cases is shown in Table III. As a group, mTBI/non-PTSD cases were significantly younger than control non-TBI/non-PTSD cases (group averages 27.4±4.1 and 35.3±10.05 years, respectively; p-value 0.05). There were no significant differences between the mTBI/non-PTSD and non-TBI/non-PTSD groups with respect to group average values for post-deployment interval (3.9±2.1 and 2.5±2.2 years, respectively; p=0.15) or years of education (13.86±2.1 and 14.4±2.2 years, respectively; p=0.59). The proportion of males in the mTBI and the non-TBI groups were 100% and 88%, respectively.

We found no significant differences in PBMC contents of ACA48, U35A, U55, or U83A in mTBI vs. non-TBI cases in the absence of PTSD comorbidity (Fig. 2A). Moreover, we found that these 4 snoRNA biomarkers have no predictive value for segregating mTBI and non-TBI cases when they are not comorbid with PTSD (Fig. 4B, C, D).

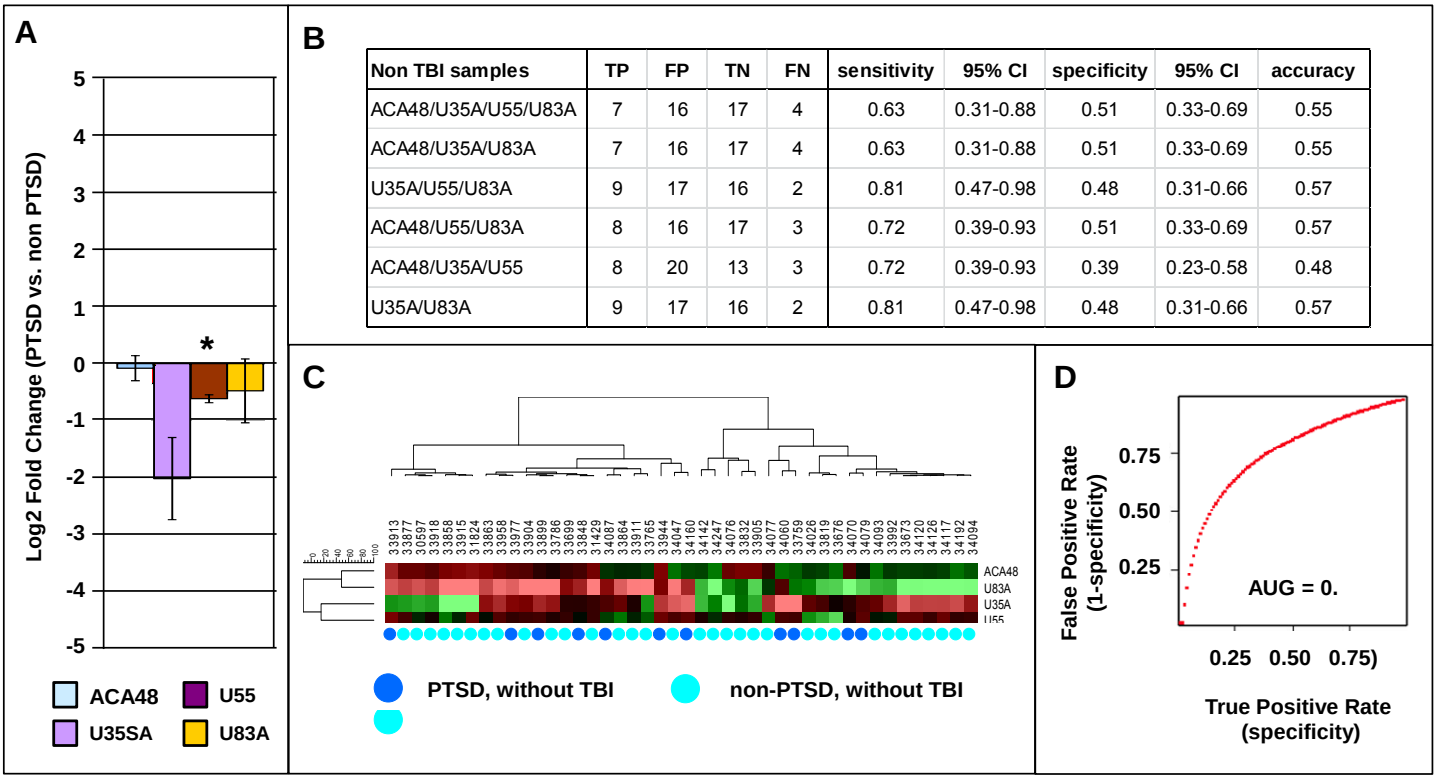


Biomarker combinations	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	5	16	17	2	0.71	0.30-0.95	0.51	0.33-0.69	0.55
ACA48/U35A/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
U35A/U55/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
ACA48/U55/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
ACA48/U35A/U55	6	19	15	1	0.85	0.42-0.99	0.54	0.36-0.72	0.52
U35A/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53

Figure 4. Candidate mTBI snoRNA biomarker expression in mTBI vs. non-TBI control cases in the absence of PTSD comorbidity. *Contents of* ACA48, U35A, U55, and U83A *in PBMC from mTBI/non-PTSD and non-TBI/non-PTSD cases were assessed by qPCR. A) t-test p-value comparison between mTBI/non-PTSD and non-TBI/non-PTSD cases. B) Unsupervised hierarchical clustering of mTBI/non-PTSD and non-TBI/non-PTSD cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples into either the mTBI/non-PTSD or the non-TBI/non-PTSD cluster. Presented is a summation table of unsupervised hierarchical clustering analyses using combinations of ACA48, U35A, U55, and U83A to correctly distinguish mTBI/non-PTSD vs. non-TBI/non-PTSD cases. (C) Heat map of an* unsupervised hierarchical clustering analysis using the combined 4 snoRNA biomarkers *demonstrates a lack of effective segregation between mTBI/PTSD and non-TBI/PTSD cases. D) ROC analysis using the combined 4 biomarkers. ROC curve plotting the percentage of correctly identified mTBI/non-PTSD cases (true positive; specificity) as a function of the percentage of non-TBI/non-PTSD cases incorrectly identified as mTBI/non-PTSD cases (false positive; 1-specificity). The calculated area under the curve (AUC) is 0.64.*

PBMC expression of ACA48, U35A, U55, and U83A in PTSD vs. non-PTSD cases in the absence of TBI: We also explored the potential effects of PTSD on regulation of the 4 validated snoRNA biomarkers. This study cohort contained 11 non-TBI/PTSD and 34 non-TBI/non-PTSD cases (Table III). There were no significant differences between the non-TBI/PTSD and non-TBI/non-PTSD groups with respect to group average values for age (33.1±11.1 and 35.4±10.1 years, respectively; p=0.53), post-deployment interval (3.6±2.5 and 2.6±2.2 years, respectively; p=0.18), or years of education (13.3±1.6 and 14.4±2.2 years; p=0.14). The proportion of males in the non-TBI/PTSD and the non-TBI/non-PTSD group were 82% and 88%, respectively.

We found no significant differences in PBMC contents of ACA48, U35A, or U83A in PTSD vs. non-PTSD cases in the absence of mTBI comorbidity (Fig. 3A). Interestingly, we did find significant down-regulation of U55 in PBMC of non-TBI/PTSD compared to non-TBI/non-PTSD. This evidence suggests that PTSD has an effect on the regulation of U55, which may contribute to the down-regulation of U55 seen in mTBI vs. non-TBI cases in the context of PTSD comorbidity (Fig. 3A, B). Nonetheless, we found that application of the 4 validated mTBI/PTSD snoRNA biomarkers, ACA48, U35A, U55, and U83A, did not have any predictive value for differentiating PTSD from non-PTSD cases in the absence of mTBI comorbidity (Fig. 5B, C, D).



Non TBI samples	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	7	16	17	4	0.63	0.31-0.88	0.51	0.33-0.69	0.55
ACA48/U35A/U83A	7	16	17	4	0.63	0.31-0.88	0.51	0.33-0.69	0.55
U35A/U55/U83A	9	17	16	2	0.81	0.47-0.98	0.48	0.31-0.66	0.57
ACA48/U55/U83A	8	16	17	3	0.72	0.39-0.93	0.51	0.33-0.69	0.57
ACA48/U35A/U55	8	20	13	3	0.72	0.39-0.93	0.39	0.23-0.58	0.48
U35A/U83A	9	17	16	2	0.81	0.47-0.98	0.48	0.31-0.66	0.57

Figure 5. Candidate mTBI snoRNA biomarker expression in PTSD vs. non-PTSD control cases in the absence of TBI comorbidity. *A) PBMC contents of* ACA48, U35A, U55, and U83A *in PTSD/non-TBI and non-PTSD/non-TBI cases, as assessed by qPCR. Bar graphs present mean and SEM values; * p<0.05, t-test p-value comparison between PTSD/non-TBI vs. non-PTSD/non-TBI cases. B,C) Unsupervised hierarchical clustering of PTSD/non-TBI and non-PTSD/non-TBI cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples to PTSD/non-TBI and non-PTSD/non-TBI clusters. Presented is a summation table of unsupervised hierarchical clustering analyses using combinations of ACA48, U35A, U55, and U83A to correctly identify* PTSD/non-TBI and non-PTSD/non-TBI cases *(B). Heat map of an* unsupervised hierarchical clustering analysis shows that a combination of *ACA48, U35A, U55, and U83A* is not effective in segregating PTSD/non-TBI vs. non-PTSD/non-TBI cases (C). *D) ROC analysis using ACA48, U35A, U55, and U83A. The calculated area under the curve is 0.75.*

Regulation of the validated mTBI/PTSD snoRNA biomarkers in animal models of blast-induced mTBI or PTSD

Recently, a blast-induced rat model mTBI was developed to demonstrate the effects of air blast exposure in OEF/OIF veterans (Elder et al., 2012). Furthermore, mice exposed to chronic social defeat are being used to model anxiety and depression symptoms seen in PTSD (Golden et al., 2011; Yang et al., 2010). Using the blast-induced rat mTBI model and the chronic social defeat mouse PTSD experimental model, we continued to explore potential cause-effect relationships between mTBI and/or PTSD and down-regulation of our validated snoRNA mTBI/PTSD biomarkers in PBMC. Among the 4 validated snoRNA mTBI/PTSD biomarkers of interest, rat sequence information is available only for ACA48. Therefore, we explored the regulation of ACA48 in rodent models of blast-induced mTBI or PTSD to gather proof-of-concept evidence that would show that mTBI and/or PTSD have implications on the regulation of the 4 snoRNA biomarkers.

We collected blood specimens from blast-treated rats ~1.3 years after their initial blast exposure to simulate the long-term post-deployment characteristics of the OEF/OIF cases in our initial biomarker discovery [12] and in our current biomarker validation studies. We found no change in the regulation of ACA48 in blood specimens from the blast-induced mTBI rat model compared to control rats (Fig. 5A), implicating that the down-regulation of ACA48 that we observed in veterans with comorbid mTBI and PTSD (Fig. 3A,B) might not be due to exposure to blast-induced mTBI. Further experimental assessments evaluating the potential impact of blast exposure on the regulation of U35A, U55, and U83A in the blast-induced mTBI rat model will have to wait for the availability of rat sequence information for these snoRNA biomarkers.

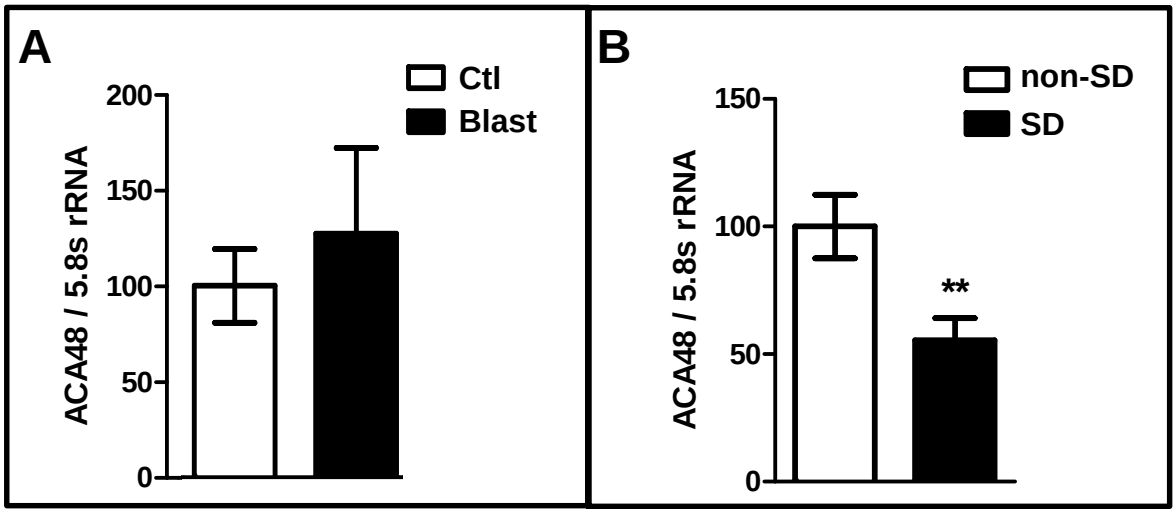


Figure 6. ACA48 snoRNA expression in blood cells from experimental rodent models of PTSD and TBI. *A blast-induced mTBI rat model [5] and a chronic social defeat (SD) mouse model of PTSD [17]) were used in these studies. Contents of ACA48 snoRNA biomarker in blood cells were analyzed by qPCR using primer sets designed for targeting rat or mouse ACA48. A) Expression of ACA48 in blood cells from rats exposed to blast-mediated TBI rats compared to control rats. Blood specimens were collected ~1.3 years after initial blast exposure. B) Expression of ACA48 in blood cells from mice subjected to SD compared to control mice. Blood specimens were collected within a short time (~24 h) after completion of the chronic SD protocol. (A, B) Bar graphs represent ACA48 content normalized to those for 5.8S rRNA using the $2^{-\Delta\Delta Ct}$ method. Levels of target small non-coding RNA were expressed relative to those in the control groups. T-test, * $p<0.05$.*

Exploring the potential influence of PTSD on the regulation of ACA48, we observed a significant down-regulation of ACA48 (by approximately 50%) in blood cells of mice exposed to chronic social defeat (SD) compared to control mice (Fig.6B). Our observation demonstrates, for the first time, that experimental PTSD is associated with altered regulation of a specific snoRNA, implicating that the presence of PTSD may contribute to the observed down-regulation of ACA48 in veterans with comorbid mTBI and PTSD. We note that our experimental evidence of down-regulation of ACA48 was based on monitoring the PTSD mouse model within a short time frame (by 24 hours) after completion of the chronic social defeat protocol. Interestingly, we found no observable changes in the content of ACA48 in PTSD vs. non-PTSD cases in the absence of TBI long after the veterans' deployment (average post deployment interval of 3.6 years) (Fig. 6A), suggesting that while PTSD may have a direct acute impact on the regulation of ACA48 in PBMC, this effect may not last over the long-term. Additional studies will be required to test longer-term impacts of PTSD on the regulation of ACA48, U35A, U55, and U83A in this PTSD mouse model.

3E. Discussion

3E1. Validation of select snoRNA biomarkers for distinguishing veteran mTBI cases with or without PTSD comorbidity:

We confirmed that 4 snoRNA species, ACA48, U35A, U55, and U83A, are significantly down-regulated in PBMC specimens from mTBI/PTSD compared to control, non-TBI/PTSD cases in two independent study cohorts. Moreover, we established the ability of these 4 validated biomarkers to distinguish cases with or without mTBI in the context with PTSD comorbidity. Identification of these snoRNA biomarkers will provide for improved detection of co-morbid mTBI and PTSD and for more sensitive measurements for clinical trials.

We observed that the 4 validated snoRNA mTBI/PTSD biomarkers were not differentially regulated among mTBI and non-TBI in the absence of PTSD comorbidity, suggesting that the presence of mTBI alone is insufficient to induce the down-regulation of ACA48, U35A, U55, and U83A seen in cases of comorbid mTBI and PTSD. Consistent with this, our ongoing studies observed no detectable change in contents of ACA48 in blood cells from a blast-induced TBI rat model.

We found significantly lower contents of U55 in PBMC from non-TBI/PTSD vs. non-TBI/non-PTSD, but no detectable changes in the regulation of ACA48, U35A, or U83A in PBMC of OEF/OIF veterans with PTSD compared to veterans without PTSD in the absence of TBI comorbidity long after their deployment. Interestingly, our ongoing studies using the chronic social defeat PTSD mouse model suggest that PTSD may lead to down-regulation of ACA48 over the short-term. This may reflect a short-term, acute response to trauma exposure, since we found no changes in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment. Additional investigations will be necessary to explore the short- and long-term contributions of PTSD on the down regulation of ACA48, U35A, U55, and U83A in veterans with comorbid mTBI and PTSD.

Our evidence suggests that the presence of mTBI alone is insufficient to induce down-regulation of these snoRNA biomarkers, but the presence of PTSD may contribute to the down-regulation of U55 and ACA48 seen in mTBI veterans with comorbid mTBI and PTSD. Our observations suggest that biological interactions between TBI and PTSD may contribute to the clinical features of mTBI with comorbid PTSD. Thus, ongoing and future investigations on mTBI mechanisms or TBI biomarkers should carefully consider their interactions with PTSD.

Most mTBI cases among veterans of the conflicts in Afghanistan and Iraq are due to blast-induced injuries. However, we do not have any information on our study cohort in regards to the type of injury—blast versus non-blast—that individual cases were exposed to. Future studies will be necessary to explore the impact of blast and non-blast TBI injuries on the regulation of these snoRNA biomarkers.

3E2. Potential relevance of the four snoRNA biomarkers to TBI/PTSD pathophysiology:

The 4 small noncoding RNA mTBI/PTSD biomarkers that we validated in our present studies are all members of the small nucleolar RNA (snoRNA) class. SnoRNAs are known for their methylation and pseudouridylation of other small noncoding RNAs, particularly ribosomal RNAs, transfer RNAs, and small nuclear RNAs (Okada et al., 1986). Methylation/pseudouridylation of small noncoding RNAs may modulate the folding of small noncoding RNAs and protect small noncoding RNAs from hydrolysis (and Lowe, 201). Recent evidence demonstrates that select snoRNA (snoRNA HBII-52) is also involved in alternative splicing of the serotonin receptor 2C (Kishore and Stamm, 2006). Changes in the regulation of snoRNAs have been associated with multiple cancers, and deletion of imprinted snoRNAs in chromosome 15q11-q13 has been associated with Prader-Willi syndrome (Sridhar et al., 2008). There is, however, little information on the potential biological activities of ACA48, U35A, U55, and U83A.

Recent evidence revealed that certain snoRNAs, referred to as sno-miRNAs, may also exhibit microRNA (miRNA) functions by interfering with translation and/or by promoting degradation of targeted mRNAs (Brameier et al., 2011). Interestingly, one of our snoRNA biomarkers, U83A, is among the sno-miRNAs reported to exert sno-miRNA activities (Brameier et al., 2011). Ongoing studies are attempting to characterize specific gene targets of U83A.

Another snoRNA biomarker validated in our present study, U35A, has been shown to be effective in modulating cellular responses to oxidative stress and inflammatory mediators. It has also been demonstrated that knockdown of U35A mitigates lipopolysaccharides-induced oxidative stress in the liver (Michel et al., 2011). While the mechanisms of action are presently unknown, these observations suggest that down-regulation of U35A in PBMC of veterans with comorbid TBI and PTSD may reduce resilience to inflammatory stress, leading to the promotion of oxidative stress conditions and contributing to mTBI and/or PTSD clinical features over the long-term.

4.KEY RESEARCH ACCOMPLISHMENTS

A. Analysis using two independent study cohorts of OIF/OEF veterans validated 4 snoRNA biomarkers (ACA48, U35A, U55, and U83A), whose down-regulation in accessible PBMC are able to dissect subjects with comorbid mTBI and PTSD from PTSD subjects without mTBI with 100% sensitivity, 81% accuracy, and 72% specificity.
B. No significant differential expression of snoRNA biomarkers was found in mTBI subjects without comorbid PTSD. Moreover, no significant differential expression for three of the biomarkers (ACA48, U35A and U83A) was found in PTSD subjects in the absence of mTBI.
C. Proof-of-concept studies exploring regulation of ACA48 in a rodent model of blast-induced mTBI demonstrated ACA48 expression in blood cells is not modulated by experimental mTBI.
D. Proof-of-concept studies exploring regulation of ACA48 in a rodent model of PTSD demonstrated lower contents of ACA48 in blood cells within 24 h of experimentally-induced PTSD. This may reflect a short-term response to trauma exposure, since we found no change in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment.
E. Collectively, our observations suggest that future investigations on mTBI biomarkers, as well as pathogenic mechanisms underlying mTBI, should carefully consider the impact of interactions with PTSD.
F. Our evidence also suggest that additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD.
G. Future studies clarifying the potential bioactivity of ACA48, U55, and U83A will provide additional insight on pathogenic mechanisms contributing to long-term clinical complications in veterans with comorbid TBI/PTSD.

5.CONCLUSION

In initial Biomarker Discovery studies we identified 13 candidate small noncoding RNA biomarkers that are differentially regulated in PBMC of mTBI compared to non-TBI control cases. In follow-up Biomarker Validation study using an independent cohort of 58 veteran cases, we explored the regulation of these candidate biomarkers in mTBI and non-TB presence or absence of PTSD comorbidity. We confirmed that 4 small nucleolar RNAs (snoRNAs), ACA48, U35, U55, and U83A, are significantly down-regulated in PBMC from veterans with mTBI and PTSD compared to non-TBI, control subjects with PTSD only. We found that the snoRNA biomarkers are able to dissect subjects with comorbid mTBI and PTSD from PTSD subjects without mTBI with 100% sensitivity, 81% accuracy, and 72% specificity. No significant differential expression of snoRNA biomarkers was found in mTBI subjects without comorbid PTSD. However, we found significantly lower U55 contents in subjects with PTSD. We explored the regulation of ACA48 in rodent models of PTSD or blast-induced mTBI to gather proof-of-concept evidence that would connect the regulation of the biomarkers and the development of mTBI or PTSD. We found no change in the regulation of ACA48 in the mTBI rat model. We did, however, find significant down-regulation of ACA48 in the PTSD mouse model 24 hours following psychological trauma exposure. This may reflect a short-term response to trauma exposure, since we found no change in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment. Additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD. Our observations suggest that biological interactions between TBI and PTSD may contribute to the clinical features of veterans with comorbid mTBI and PTSD. Future investigations on mTBI mechanisms or TBI biomarkers should consider their interactions with PTSD.

6.PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Publications:

1. Pasinetti, G.M., Fivecoat, H., Ho, L. (2010) Perspectives in traumatic brain injury. Psychiatr Clin North Am 33(4):905-913. PMID: 21093685

2. Pasinetti GM, Ho L, Dooley C, Abbi B, Lange G (2012) Select non-coding RNA in blood components provide novel clinically accessible biological surrogates for improved identification of traumatic brain injury in OEF/OIF Veterans. Am J Neurodegener Dis 1: 88-98. PMID: 22737634.

3. Ho, L., Lange, G., Zhao, Wang, J., Rooney, R., Patel D.H., Fobler, M., Helmer, D.A., Elder, G., Shaughness, M.C., Ahlers, S.T., Russo, S.J., Pasinetti, G.M. (2014) Select small nucleolar RNAs in blood components as novel biomarkers for improved identification of comorbid traumatic brain injury and post-traumatic stress disorder in veterans of the conflicts in Afghanistan and Iraq. (Submitted).

7.INVENTIONS, PATENTS AND LICENSES

Nothing to report

8.REPORTABLE OUTCOMES

We identified four snoRNA biomarkers (ACA48, U35A, U55, and U83A), whose down-regulation in accessible PBMC are able to dissect subjects with comorbid mTBI and PTSD from PTSD subjects without mTBI with 100% sensitivity, 81% accuracy, and 72% specificity.

9.OTHER ACHIEVEMENTS

Nothing to report

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11.APPENDICES

1. Pasinetti, G.M., Fivecoat, H., Ho, L. (2010) Perspnalized medicine in traumatic brain injury. Psychiatr Clin North Am 33(4):905-913. PMID: 21093685
2. Pasinetti GM, Ho L, Dooley C, Abbi B, Lange G (2012) Select non-coding RNA in blood components provide novel clinically accessible biological surrogates for improved identification of traumatic brain injury in OEF/OIF Veterans. Am J Neurodegener Dis 1: 88-98. PMID: 22737634.
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Personalized Medicine in Traumatic Brain Injury

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KEYWORDS

• Postconcussive traumatic brain injury • MicroRNA • Biomarker
• Personalized medicine

A fine line exists between a trivial head blow and one that affects the brain to produce a mild traumatic brain injury (TBI). The widely varying clinical effects of mild brain injury provide grounds for substantial physical, cognitive, and psychosocial disability. To this end, an opinion may be requested of a rehabilitation specialist, neurologist, psychiatrist, or other physician seeing the patient for the first time months, or even years, after the traumatic event. Ideally, for an evidence-based diagnosis of a postconcussive disorder to be made, the following 4 factors are required: (1) a credible mechanistic force applied to the brain, sufficient to cause microstructural or at least molecular injury to the brain; (2) acute clinical effects that are both recognizable and verifiable; (3) partitioning of nonspecific or confounding symptoms and findings arising independently of the brain injury; and (4) a discernible end point of recovery or disability.[1] A strong need exists for the creation of minimum objective requirements and guidelines based on these precepts to determine whether a mild brain injury has occurred resulting in postconcussive symptoms. A second area in need of research is in establishing whether "persistent postconcussive syndrome" exists as a distinct biological entity. Yet a third area in need of research is in elucidating criteria that establish chronic traumatic encephalopathy as a unique cognitive disorder. As discussed in this article, these important questions may be addressed with the aid

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of current genomic technologies providing selective molecular signatures of disease ahead of time before clinical symptoms become available.

TOWARD PERSONALIZED MEDICINE IN TBI

The identification and characterization of "molecular fingerprints" of postconcussive disorders is critical in establishing that symptoms are related to a mild TBI. Posttraumatic symptoms can develop in the hours and days after a mild TBI and may include insomnia, stress, headaches, pain, and mood disturbances.[1] The availability of mild TBI biomarkers offer a potential tool to ascertain that injury has occurred, and may provide a marker for prognosis and a guide for treatment response. Recent evidence identified microRNAs (miRNA) as important regulators of cellular function,[2,3] and that deregulation of select miRNA network in the brain has been associated with neurodegenerative disorders.[4–8] Moreover, there is accumulating evidence that supports the feasibility of using miRNA fingerprints from peripheral blood mononuclear cells (PBMC) to identify clinically accessible molecular indexes (biomarkers) of neurological disorders, with the potential for developing these biomarkers as practical diagnostic biological surrogates for the onset and clinical progression of a neurodegenerative disorder (see later discussion). Thus, it has been hypothesized that comparable strategies could be used to identify and characterize molecular indexes (biomarkers) capable of predicting the risk for the development of postconcussive symptoms or chronic traumatic encephalopathy (CTE). The development of specific biomarkers for mild TBI holds the potential to provide insights on novel strategies to prevent or minimize post-TBI sequelae.

An emerging health care challenge exists in the United States for how to best care for the increasingly large number of military veterans, particularly those involved in Operation Enduring Freedom and Operation Iraqi Freedom (OEF/OIF), who have been exposed to concussive injuries. The scientific development of molecular indexes that can signal complications of mild TBI, before the appearance of clinical signs and symptoms, is in line with the health care strategy of the United States Departments of Veteran Affairs and Defense, which focuses on prevention and early intervention rather than reactions to advanced stages of diseases.

LACK OF ESTABLISHED THERAPY FOR COMBAT-RELATED TBI

Based on the presumption that TBI induces structural brain damage and that recovery depends on adaptation, treatment of postconcussive symptoms to date has focused on pharmacotherapy to target individual symptoms, on cognitive rehabilitation mainly to help attention and memory, and on compensatory strategies through cognitive-behavioral therapies.[9] Although evidence supports the efficacy of cognitive-behavioral therapies for treating TBI in the civilian population,[10–13] many clinical trials have failed to reduce disability.[14,15] Moreover, no consensus exists on how to best treat combat-related TBI among OIF/OEF military personnel during and/or following their military deployment.

The mechanisms underlying mechanical and blast-related TBI may differ in some ways, but they share important pathophysiological features. Of note, a common feature of mechanical and blast injury is diffuse axonal injury caused by angular forces, which induce the shearing or stretching of axons.[9,16,17] This shearing results in impaired axonal transport and focal axonal swellings,[9] leading to impaired neurological functions. This and other similarities between the pathophysiologies of mechanical and blast-related TBI[9] suggest that information gathered from TBI in the civilian population may also be relevant to combat-related TBI.

VARIABLE SUSCEPTIBILITY OR RESILIENCY AMONG INDIVIDUALS EXPOSED TO MILD TBI

Mild TBI patients from both civilian and military populations exhibit varying clinical symptoms with minimal to profound impact on their daily functioning. This variation is reflected in a recently published mental health outcome survey of 105 OIF combatants diagnosed with mild TBI.[18] In this study, health status of eligible deployed military personnel was assessed by thorough review of their clinical records, and both diagnostic and injury severity scores were assigned.[19,20] Twenty-nine cases (27.6%) had presenting mental health problems (International Classification of Diseases [ICD-9] 290–319), with 17 out of the 29 cases presenting mood disorder (ICD-9 296, 300.4, 301.13, 311) and anxiety disorders (ICD-9 300–300.02, 300.21–300.29, 300.3, 308.3, 308.9, 309.81). Thus, clinical repercussions of mild TBI vary significantly among individuals.

The reason why mild TBI is associated with varying clinical symptoms among different individuals in response to mechanical or blast trauma among civilians or combat veterans is currently unknown. The possibility that miRNA from PBMC may reflect phenotypic changes in response to the mild TBI represents an unprecedented opportunity to explore the biomarker expressions that underlie the range of symptom expression observed after TBI.

POTENTIAL INTERRELATIONSHIPS BETWEEN TBI AND ALZHEIMER DISEASE–TYPE COGNITIVE DETERIORATION

Current literature suggests that TBI may be a risk factor for dementia and that they may be a cumulative risk from repeat events.[21] Although the early literature suggested that neuropathological mechanisms known to underlie Alzheimer disease (AD) dementia may have been similar to the one occurring in post-TBI patients, new evidence suggests that CTE is characterized by neuropathological findings that are distinct from the findings in AD.[22] The pathologic findings in these patients are believed to be responsible for cognitive decline seen in some patients with histories of TBI. The 2 characteristic neuropathologies of AD are the abnormal accumulation and deposition of beta-amyloid (Aβ) peptides and tau proteins in the brain. Evidence from humans[22–24] and experimental animal models[25] has also revealed abnormal accumulations of Aβ peptides and tau proteins in the brain and in cerebral spinal fluids following TBI; however, the location and the distribution of tau protein in TBI patients are different from the characteristic pattern seen in AD. Of note, there is some evidence suggesting that elevation of plasma tau levels has been associated with increasingly severe outcomes of TBI.[26] Thus, AD-related neuropathological mechanisms may contribute to cognitive dysfunction in TBI. Consistent with this, a recent study demonstrated that cognitive and motor deficits following TBI in experimental mouse models could be ameliorated by blocking either β- or γ-secretase, the 2 enzymes necessary for the generation of Aβ peptides from the amyloid precursor protein.[27] Molecular fingerprinting studies may lead to a better understanding of why certain individuals who have sustained a mild TBI may be susceptible to developing dementia.

FROM VISUAL PHENOTYPE TO HIDDEN MOLECULAR SIGNATURES: PERSONALIZED MEDICINE AND MILD TBI

In modern medicine, physicians and scientists alike have viewed and interpreted disease at the “visual” level, namely the level of the organism, the organ, and more

recently, the tissue. With the advent of genomics and proteomics technologies, personalized medicine offers the promise and potential of uncovering the largely "unseen" details of disease causality, onset, and progression. A broad aim of personalized medicine is to use a molecular characterization approach to create a better system for disease classification. This work is anticipated to lead to earlier interventions and more specific treatments according to an individual's specific biochemical fingerprint; this is in stark contrast to current medical practice that is focused on the present state of health or disease. The differences between these 2 approaches may be best understood by likening them to one's view of an iceberg, which historically has been defined only by the very small part that is visible and almost inconsequential in relation to the predominant part that is below the surface yet defines the essence of the iceberg's existence (**Fig. 1**). Likewise, until now scientists and physicians have been limited to viewing and interpreting the most easily visible aspects of disease (at organism, organ, and tissue levels); by contrast, personalized medicine promises to reveal a far deeper and more comprehensive view of the largely unseen details of disease causality, onset, and progression by enabling them to view a disease from its onset and to monitor its progression at the molecular and cellular levels. Thus, the clinical presentations of patients who have sustained a mild TBI is conceptually comparable to the "tip of the iceberg" when the major impacts of the disease remain unseen. However, and most importantly, our understanding of disease is enhanced by the ability to view the "hidden, submerged unseen mass" of a given condition from its onset or, in other words, by the ability to monitor disease progression at the molecular level through the use of novel genomic technology (eg, miRNA at the cellular-molecular level, as depicted in **Fig. 1**). To date, some specific examples of personalized

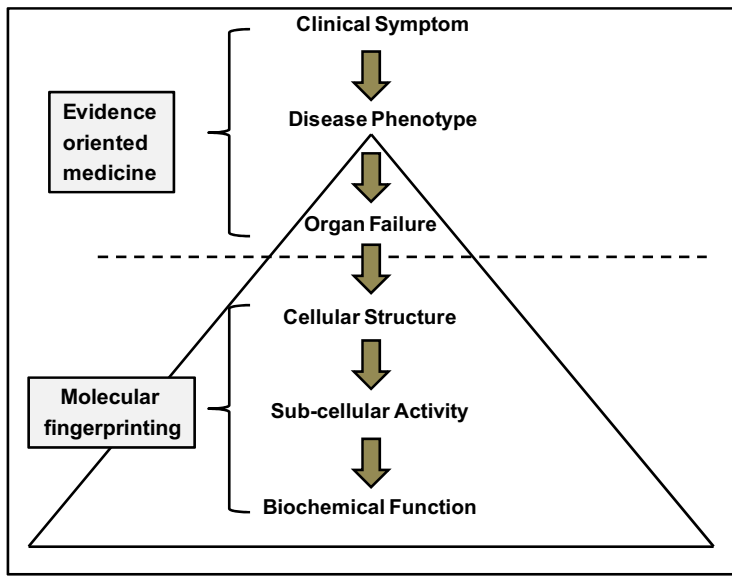

Fig. 1. Schematic subdivision of postconcussive syndrome as clinical and biochemical entity at various functional levels within an organism following mild TBI. The top bracket embraces those levels of disease for which traditional "evidence-oriented" medicine has been successful in the last 2 centuries (tip of the iceberg). The lower bracket embraces the extended region accessible by personalized medicine attempting to define the disease at cellular/biochemical levels.

medicine approaches, including the recent use of miRNA, have been utilized to benefit diagnosis and treatment of diseases in patients.

BIOLOGY OF miRNA AND THE SILENCING OF GENE EXPRESSION: IMPLICATIONS FOR UNDERSTANDING NEURODEGENERATIVE DISORDERS

The miRNA species are naturally present in 21- to 22-nucleotide base RNAs, functioning to silence their target genes' messenger RNAs either by binding to the coding region and promoting cleavage and degradation, or by inhibiting their translation by binding at the 3′-untranslated region (UTR).[28,29] New evidence points to miRNA as an important index of gene regulation.[2,3] An individual miRNA may target many gene transcripts that include specific target sequences; at the same time, a target gene's messenger RNA may possess multiple target sequences vulnerable to control by multiple miRNAs.[30] This versatility in miRNA silencing provides cells with an exquisite ability to programmatically shift rapidly among various signaling pathways. This exciting emerging discovery of the suppressing action of miRNAs does not negate the importance of other biological processes involved in controlling gene expression such as promoter-based transcriptional regulation, changes in DNA structure, or binding interaction to nuclear proteins. Most importantly, it serves as a complementary system to transcriptional control and modifying the intensity of individual gene expression in order to obtain a spectrum of its signal manifestation rather than an all-or-none outcome. miRNAs have recently emerged as key regulators of complex temporal and spatial patterns of gene/protein expression changes and, thereby, synaptic and neural plasticity.[28,31] Accumulating evidence suggests that selectively deregulated miRNA expression networks in the brain could mechanistically contribute to the onset and/or progression of neurodegenerative disorders, including AD,[4–6] Huntington disease,[7] and Parkinson disease.[8] These findings suggest that there are underlying genetic mechanisms that affect the brain's response to injury and, if true, would explain the individual variability in response to TBI.

EVIDENCE SUPPORTING THE FEASIBILITY OF USING CLINICALLY ACCESSIBLE MOLECULAR INDEXES FOR DEVELOPING PERSONALIZED MEDICINE APPROACHES IN MILD TBI PREVENTIVE STRATEGIES

There is increasing interest in exploring the prognostic values of plasma molecular signatures to predict clinical outcome following TBI. Indeed, high serum levels of S100B,[32] neuron-specific enolase,[32,33] glial fibrillary acidic protein,[32] and tau[26,34] following TBI have been linked to poor long-term outcome. Detecting abnormal levels of these markers may trigger interventions aimed at preventing future disease. Two genetic tests currently on the market can identify disease susceptibility and guide preventive care. First, a recent molecular predictive indicator of disease, which has received wide attention, is the test for BRCA1 and BRCA2, 2 genetic variants that indicate a hereditary propensity for breast and ovarian cancers.[35] Women with BRCA1 or BRCA2 genetic risk factors have a 36% to 85% lifetime chance of developing breast cancer, compared with a 13% chance among the general female population. The BRCA1 and BRCA2 genetic tests can be used to guide preventive measures, such as increased frequency of mammography, prophylactic surgery, and chemoprevention. Second, the treatment of early-stage breast cancer in women may be transformed, for example, by several assays in development that scan a panel of genes correlated with risk of disease recurrence and response to therapy.[36] One such assay now being used in clinical settings is Onco*type* DX (Genomic Health, Redwood City, CA), which analyzes the expression of 21 genes.[37]

It can be hypothesized that similar approaches may soon be available for identifying patients at risk for developing postconcussive disorders including CTE. The information provided by such a test would be fundamental to supporting both disease treatment and monitoring decisions, based on the foreknowledge of disease progression, time to event, and likelihood of treatment benefit.[38] The degree of success in individualizing medicine in TBI and neuropsychological complications, however, will depend on the degree to which molecular aspects of the disease can be elucidated and measured. Feasibility studies of Parkinson disease suggest that miRNA technology holds the potential to provide a novel, clinically accessible tool for preventative medicine strategies in mild TBI (Pasinetti and Ho, personal Communication, 2008). The authors' laboratory has demonstrated for the first time that the regulation of specific peripheral molecular indices (eg, miRNA in PBMC) is associated with the onset and/or progression of Parkinson disease. In recent ongoing studies, higher contents of 3 PBMC miRNA biomarkers have been found in the substantia nigra specimens of Parkinson patients than in normal cases. The authors' success in identifying peripheral biomarkers for Parkinson disease strongly supports the hypothesis that a comparable genomic technological approach could be used in the identification of biomarkers for the development of postconcussive disorders.

Thus, the use of high-throughput miRNA assays combined with reverse transcription–polymerase chain reaction validation studies might lead to the identification and characterization of molecular fingerprints (ie, biomarkers) associated with TBI phenotypic expression among individuals with mild TBI. These clinically accessible biomarkers provide the opportunity to test whether postconcussive symptoms of CTE could be predicted at molecular levels in patients with TBI prior to definitive clinical diagnosis.[1] Moreover, the availability of TBI biomarkers may provide a better understanding of the molecular mechanisms underlying resiliency to TBI clinical complications, and may provide predictive biomarkers of resiliency/susceptibility to TBI clinical complications. Ultimately, clinically accessible TBI biomarkers may provide the basis for developing a novel personalized medicine approach to the treatment of patients with mild TBI. Based on evidence from the authors' laboratory that miRNA species in PBMC may reflect neurodegenerative disorders (eg, Parkinson disease) at early, preclinical phases of the disease, it can be posited that PBMC may provide an ideal and clinically accessible "window" into the brain. Thus, it is possible that changes in the expression profile of clinically accessible biomarkers, such as miRNA in PBMC, may reflect molecular alterations following TBI that contribute to the onset and progression of TBI phenotypes.

SUMMARY: IMPACT OF THE CLINICALLY ACCESSIBLE BIOMARKERS IN MILD TBI

According to existing data, more than 1.5 million people are treated in hospitals for TBI each year in the United States, 75% of whom for mild TBI.[38] These injuries may cause long-term or permanent impairments and disabilities. Many people with mild TBI have difficulty returning to routine, daily activities and may be unable to return to work for many weeks or months. In addition to the human toll of these injuries, mild TBI costs the nation nearly $17 billion each year.[38] These data, however, likely underestimate the problem of mild TBI for several reasons: first, no standard definitions exist for mild TBI and mild TBI-related impairments and disabilities. The existing Centers for Disease Control and Prevention (CDC) definition for TBI surveillance is designed to identify cases of TBI that result in hospitalization, which tend to be more severe. Mild TBI is often treated in nonhospital settings, or is not treated at all. Few states conduct emergency department–based surveillance, and current efforts do not capture data about

persons with mild TBI who receive no medical treatment. In addition, neither hospital-based nor emergency department–based data can provide estimates of the long-term consequences of mild TBI. In response to concerns about this public health problem, Congress passed the Children's Health Act of 2000, which required the CDC to determine how best to measure the incidence (ie, rate at which new cases of mild TBI occur) and the prevalence (ie, proportion of the United States population at any given time that is experiencing the effects) of mild TBI, and to report the findings to Congress.[39,40] To that end, the CDC formed the Mild Traumatic Brain Injury Work Group to determine appropriate and feasible methods for assessing the incidence and prevalence of mild TBI in the United States.

The development of clinically accessible biomarkers, such as specific miRNA biomarker species in PBMC, will serve as independent, objective biological surrogates to help predict complications of TBI in a population (such as veterans who sustained a concussive injury) at high risk for developing postconcussive disorders. This particular aim is in line with the Children's Health Act of 2000 and CDC mission. This new approach will make it possible to use molecular indexes that signal the risk of developing postconcussive sequelae or its presence before clinical signs and symptoms appear, and has the potential to have a major impact in preventative measures among combat veterans and in the American civilian public sector.

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Original Article

Select non-coding RNA in blood components provide novel clinically accessible biological surrogates for improved identification of traumatic brain injury in OEF/OIF Veterans

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Abstract: This study was designed to identify clinically accessible molecular biomarkers of mild traumatic brain injury (mTBI) that could be used to help identify returning Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) Veterans who are suffering from the effects of mTBI. While analyzing the expression profile of small non-coding RNAs in peripheral blood mononuclear cells (PBMCs) from an OEF/OIF veteran study cohort using a high throughput array chip platform, we identified 18 candidate small non-coding RNA biomarkers that are differentially regulated in PBMCs of mTBI compared to non-TBI control cases. Independent quantitative real-time polymerase chain reaction assays confirmed that 13 of these candidate small RNA biomarker species are, indeed, significantly down-regulated in PBMCs of mTBI compared to non-TBI control veteran cases. Based on unsupervised clustering analysis, we identified a 3-biomarker panel which was most able to distinguish mTBI from non-TBI control veteran cases with high accuracy, selectivity and specificity. The majority of mTBI cases in our biomarker study were co-morbid with Post-Traumatic Stress Disorder (PTSD), and thus our non-TBI control cases were selected to match PTSD diagnoses. Therefore, our identified panel of 3 small RNA biomarkers likely represents a biological index selective for mTBI. Outcomes from our studies suggest that additional applications of the clinically accessible small non-coding RNA biomarkers to current diagnostic criteria may lead to improved mTBI detection and more sensitive outcome measures for clinical trials. Future studies exploring the physiological relevance of mTBI biomarkers will also provide a better understanding of the biological mechanisms underlying mTBI and insights into novel therapeutic targets for mTBI.

Key words: Mild traumatic brain injury (mTBI), biomarkers, microRNA (miRNA), post-traumatic stress disorder (PTSD)

Introduction

Traumatic brain injury (TBI) is a condition often identified among Veterans deployed to the Persian Gulf region in support of Operation Enduring Freedom (OEF) or Operation Iraqi Freedom (OIF). TBI is caused by one or more concussive insults to the head or a penetrating head injury that disrupts the normal functions of the brain, leading to either transient or chronic impairments in physical, cognitive, emotional and behavioral functions [1-6]. In OEF/OIF Veterans, TBI is largely the result of concussive injuries from blast-producing weaponry [7]. Veterans exposed to blasts from prior conflicts have shown evidence of mild TBI (mTBI) and attention difficulties when compared to similar Veterans without blast exposure. Mild TBI can be difficult to diagnose and, when coupled with psychological illness, can be either misdiagnosed or missed altogether. Traditionally, physicians and scientists have viewed and interpreted diseases at the 'visual' clinical level. However, with the advent of genomics and proteomics technologies, personalized medicine offers the promise and potential of uncovering the largely 'unseen' details of disease causality, onset, and progression.

New evidence has highlighted defects in neural

circuits and synapses, and the plastic processes controlling these functions, in TBI [8-13]. While gene products relevant to these processes are expressed in the brain, some of these genes are also expressed in circulating blood cells, such as peripheral blood mononuclear cells (PBMCs) [14-17]. Consistent with this, recent studies have illustrated that PBMC-associated biomarkers may provide insights into the pathogenesis of neurological disorders such as Alzheimer's disease and may be used to monitor disease diagnosis and progression [18,19]. Thus, PBMCs may also provide an ideal clinically accessible "window" into the brain, reflecting molecular alterations following TBI which might contribute to the onset and progression of clinical TBI phenotypes.

Small non-coding RNAs, including microRNA (miRNA) and small nucleolar RNA (snoRNA), are increasingly recognized for their roles in the regulation of cellular processes in health and disease [20]. Select small non-coding RNAs, particularly miRNA, have been implicated in neurological disorders [21-23]. It is possible that miRNA and other small non-coding RNAs might contribute to the onset and/or progression of clinical complications following TBI [24]. Exploring the feasibility of identifying clinically accessible TBI biomarkers, we identified select small non-coding RNA fingerprints from clinically accessible PBMCs that may be used as independent biological indexes of mTBI in OEF/OIF Veterans. Outcomes from our studies suggest that additional applications of the clinically accessible small non-coding RNA biomarkers to current diagnostic criteria may lead to improved mTBI detection and more sensitive outcome measures for clinical trials.

Materials and methods

18 OIF and OEF Veterans (9 mTBI and 9 non-mTBI control cases) were recruited by The War Related Illness and Injury Study Center (WRIISC), Department of Veterans Affairs, New Jersey Health Care System (DVANJHCS), East Orange, NJ. Male and female participants were included if they were between 18-75 years of age and completed a clinical evaluation at the New Jersey WRIISC. Participants were included regardless of their mTBI history. Cases with inter-current infections or inflammatory-related conditions were excluded. Participants were classified as having a history of mTBI if they positively endorsed at least one of 4 items on the Veteran traumatic brain injury screening tool (VAT-BIST) [25] and had a score at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS) [26] . Classification criteria for Control cases included a negative VAT-BIST score and a RBANS score less than one standard deviation below the norm.

Demographic information for individual mTBI and non-mTBI cases is presented in **Table 1**. The average age of mTBI and non-mTBI cases used in our interim Biomarker Discovery study was, respectively, 31.6±7.0 and 29.8+8.2 years. The interval between Veterans' last deployment and recruitment into this study was 3.9±2.7 and 2.6±2.1 years for the mTBI and non-mTBI group, respectively. The mTBI group had an average of 13.3±1.3 years of education and the non-mTBI group had an average 13.0±2.4 years of education. There was no significant difference in age, deployment interval or years of education between the mTBI and the non-mTBI groups (t-test assessments of mTBI versus non-mTBI groups: p-values of 0.59 for age, 0.30 for deployment interval, and 0.72 for duration of education). The proportion of males in the mTBI and the non-mTBI group was, respectively, 78% and 67%. Lastly, 89% of the mTBI veteran cases used in our study were co-morbid with post-traumatic stress disorder (PTSD), based on a PTSD diagnosis criterion of having a score of 50 or more in the PTSD Checklist - Civilian Version [27]. Thus non-mTBI cases were selected to match for PTSD, with 78% of cases in the non-mTBI control group diagnosed with PTSD.

PBMC isolation

Blood specimens were collected by venipuncture and drawn into BD Vacutainer CPT Cell Preparation Tubes. PBMCs were isolated from freshly collected blood specimens following manufacturer's instructions (Becton, Dickinson and Company) and were stored at -80 ^{o}C until use.

RNA preparation and high throughput analysis of small non-coding RNAs

Total RNA was isolated from approximately 10-50 mg of PBMCs using RNA STAT-60 according to the manufacturer's instructions (Tel-Test,

Table 1. Demographic characteristics of mTBI and non-mTBI control cases we used in our interim Biomarker Discovery study. Mild TBI classification is based on positive endorsement of the VA traumatic brain injury screen (VAT-BIS) and a score of at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS). Non-mTBI case classification is based on negative endorsement of VAT-BIS and a RBANS score of less than one standard deviation below the norm. PTSD diagnosis is based on a score of 50 or more on the PTSD Checklist – Civilian Version. Average age: mTBI group, 31.6±7.0 yrs; non-mTBI group, 29.8±8.2 yrs. Interval between their last deployment and recruitment into this study: mTBI group, 3.9±2.7 yrs; non-mTBI group 2.6±2.1 yrs. Average duration of education: mTBI group, 13.3±1.3; non-mTBI group, 13.0±2.4 yrs. Percentage of males: mTBI group, 78%; non-mTBI group, 67%. Percent of cases with co-morbid PTSD: mTBI group, 89%; non-mTBI group, 78%.

Case	mTBI/Ctl	Age	Gender	Ethnicity	Interval (yrs) since last deployment	Education (yrs)	Comorbidity PTSD
31529	mTBI	38	Male	Black, non-Hispanic	3.0	14	Yes
33297	mTBI	41	Male	Native American	4.3	12	Yes
33825	mTBI	31	Male	Black, non-Hispanic	3.4	16	Yes
33828	mTBI	42	Male	Black, non-Hispanic	0.7	14	Yes
33888	mTBI	27	Female	White Hispanic	4.5	14	Yes
33931	mTBI	23	Male	White Hispanic	1.2	12	Yes
33947	mTBI	25	Female	Black, non-Hispanic	2.8	13	No
33881	mTBI	32	Male	White Hispanic	10.2	13	Yes
33811	mTBI	27	Male	Black, non-Hispanic	4.8	12	Yes
31705	Non TBI Ctl	27	Male	Black, non-Hispanic	3.4	12	No
33565	Non TBI Ctl	25	Male	White Hispanic	1.1	12	Yes
33578	Non TBI Ctl	30	Female	White Hispanic	4.3	16	Yes
33596	Non TBI Ctl	35	Male	White Hispanic	3.8	16	Yes
33598	Non TBI Ctl	49	Male	White Hispanic	0.7	9	Yes
33821	Non TBI Ctl	26	Female	White Hispanic	0.2	16	No
33834	Non TBI Ctl	24	Male	White Hispanic	2.8	12	Yes
33913	Non TBI Ctl	22	Female	Black, non-Hispanic	6.5	12	Yes
33930	Non TBI Ctl	30	Male	Black, non-Hispanic	1.0	12	Yes

Friendswood, TX, USA). Immediately prior to RNA labeling, the purity and concentration of RNA samples were determined from $OD_{260/280}$ readings using a dual beam UV spectrophotometer and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's instructions.

Total RNA was directly modified and labeled using the FlashTag™ HSR Biotin RNA Labeling Kit according to the manufacturer's instructions (Genisphere, Hatfield, PA). Verification of biotin labeling was obtained by an enzyme-linked oligoabsorbant assay (ELOSA) using Immobilizer™ Amino – 8 well strips (Nunc/Thermo Fisher Scientific, Rochester, NY, USA) according to instructions supplied by Genisphere. Labeled cRNA

(1.0 μg) was hybridized for 16hr at 48°C to Affymetrix microRNA v1.0 arrays (Affymetrix, Santa Clara, CA, USA), which contain probe sets for 1,500 small non-coding RNAs, including microRNA (miRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), and 5.8S ribosomal RNA (rRNA). Array content was derived from the Sanger miRBase miRNA database v11 (April 15, 2008, http://microrna.sanger.ac.uk), snoRNABase (www.snorna.biotoul.fr) and the Ensembl Archive (www.ensembl.org). Arrays were washed and stained on a Fluidics Station 450 (Affymetrix) according to the manufacturer's recommended procedures. The arrays were stained with phycoerythrein-conjugated streptavidin (Invitrogen/Life Technologies, Carlsbad, CA, USA) and the fluorescence intensities determined using a GCS 3000 7G high-resolution confocal laser scanner and AGCC software (Affymetrix). The scanned images were analyzed with the miRNA QC tool (Affymetrix) using RMA global background correction, quantile normalization and median polish summarization to generate quantified data (as recommended by Genisphere). Quality control metrics for arrays included normalized signal values > 1000 for five spike-in control oligo probe sets (Genisphere).

Small RNA probe sets exhibiting significant differential expression (SDE) were identified using the following steps in GeneMaths XT (Applied Maths, Austin TX): 1) Probed sets with array detection p-values ≤ 0.05 for all samples in at least one experimental group were selected for further analysis, 2) Performed Discriminant Analysis (DA) and determined the largest percentage of remaining probe sets that permitted correct group assignment of samples in unsupervised hierarchical clustering by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) based on cosine correlation of row mean centered log2 signal values; this was the top 50%-tile, 3) In the DA top 50%-tile, selected probe sets with absolute signal log2 fold changes ≥ 1.0 and independent t-test p-values ≤ 0.05 adjusted for multiple testing error by the Benjamini-Hochberg false-discovery rate (FDR) correction method [28]. Unsupervised hierarchical clustering of probes sets and heat map generation were performed in GeneMaths XT following row mean centering of log2 transformed MAS5.0 signal values; probe set clustering was performed by the UPGMA method using Cosine correlation as the similarity metric. For comparative purposes, clustered heat maps included probe sets for spike-in controls (Genisphere), or endogenous small RNAs exhibiting: 1) Array detection p-values ≤ 0.05, and 2) either a) a log2 signal value standard deviation ≤ 0.025 for all samples or b) in the DA top 50%-tile with an FC > 1.3 in the opposite direction of the selected SDE profile.

Confirmatory quantitative Real-Time Polymerase Chain Reaction (qPCR) studies

We identified specific target sequences for each of the 18 candidate small RNA biomarkers (**Table 2**). Based on the sequence information, qPCR primer sets specific for each of the biomarkers were custom-designed and synthesized commercially by Applied Biosystems (Carlsbad, CA). One microgram of total RNA was used to prepare complementary DNA (cDNA) libraries using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California) in a total volume of 20 μL. Data were normalized relative to those for the 58S ribosomal RNA. Levels of targeted small non-coding RNA were expressed relative to those in control groups using the $2^{-\Delta\Delta Ct}$ method [29].

Results

Using the Affymetrix Human gene 1.0 ST Array chip as a high-throughput platform, we analyzed the expression profile of 1500 small non-coding RNAs in our human PBMC specimens from the mTBI and non-mTBI cases in our OEF/OIF Veteran study cohort. We detected a total of 428 small RNA species from our PBMC specimens: 190 miRNAs, 220 snoRNAs, 8 small cytoplasmic RNAs and 10 ribosomal RNAs. In an initial exploratory data analysis, we conducted a principal component analysis of all signals detected to assess the potential value of the data sets to segregate mTBI and non-mTBI cases. The analysis revealed that data sets from all cases can be clustered into an mTBI or non-mTBI group, with the exception of mTBI case #33811, which is plotted far from the mTBI and non-mTBI clusters (**Figure 1**). Results from the principal component analysis suggested that the dataset generated from case #33811 was an outlier. Based on this and the fact that the quality of RNA extracted from this case was poor (not shown), we excluded case #33811 from subsequent statistical analyses.

Table 2. Targeted nucleic acid sequences selected for qPCR analysis. Shown are names of the 18 candidate small RNA biomarkers and the corresponding targeted nucleic acid sequence used for the construction of selective probe systems for qPCR studies. Also shown is a target sequence for 58SrRNA, which we selected as an internal control for qPCR studies.

Small RNA name	Target Sequence
U8	ATCGTCAGGTGGGATAATCCTTACCTGTTCCTCCTCCGGAGGGCAGATTAGAACATGAT-GATTGGAGATGCATGAAACGTGATTAACGTCTCTGCGTAATCAGGACTTGCAACACCCTGATTGCTCCTGTCTGATT
U58B	CTGCGATGATGGCATTTCTTAGGACACCTTTGGATTAATAATGAAAACAACTACTCTCTGAGCAGC
U27	ACTCCATGATGAACACAAAATGACAAGCATATGGCTGAACTTTCAAGTGATGTCATCTTACTACTGAGAAGT
U83A	GCTGTTCGTTGATGAGGCTCAGAGTGAGCGCTGGGTACAGCGCCCGAATCGGACAGTGTA-GAACCATTCTCTACTGCCTTCCTTCTGAGAACAGC
HBII-289	ACTGAGGAATGATGACAAGAAAAGGCCGAATTGCAGTGTCTCCATCAGCAGTTTGCTCTC-CATGGGCACACGATGACAAAATATCCTGAAGCGAACCACTAGTCTGACCTCAGT
U55	GTGTATGATGACAACTCGGTAATGCTGCATACTCCCGAGTGCGCGGTGGGGAAGCCAACC-TTGGAGAGCTGAGC
HBII-239	TGTGTGTTGGAGGATGAAAGTACGGAGTGATCCATCGGCTAAGTGTCTTGTCACAATGCT-GACACTCAAACTGCTGACAGCACACG
U38B	TCTCAGTGATGAAAACTTTGTCCAGTTCTGCTACTGACAGTAAGTGAAGATAAAGTGTGTCTGAGGAGA
U56	CCACAATGATGGCAATATTTTTCGTCAACAGCAGTTCACCTAGTGAGTGTTGAGACTCTGGGTCTGAGTGA
U15B	CTTCAGTGATGACACGATGACGAGTCAGAAAGGTCACGTCCTGCTCTTGGTCCTTGTCAG-TGCCATGTTCTGTGGTGCTGTGCACGAGTTCCTTTGGCAGAAGTGTCCTATTTATTGATCGATTTAGAGGCATTTGTCTGAGAAGG
U35A	GGCAGATGATGTCCTTATCTCACGATGGTCTGCGGATGTCCCTGTGGGAATGGCGACAAT-GCCAATGGCTTAGCTGATGCCAGGAG
ACA48	TGTCCCTGACCTGGGTAGAGTGGCATCTGGTTGGTGATGCCCATCTCATATCAGCCAGG-GACAAAGCAACTCCTTGTTCATCCCAGCTTGGCTTTTGATCCGTGCCCATGCCTGGTTCATGCCTTGGACACATAG
U91	TGGCCGATGATGACGAGACCACTGCGCAATCTGAGTTCTGGGAACCAGGTGATGGAGTAT-GTTCTGAGAACAGACTGAGGCCG
ENSG00000199941	TCTTAGTGACATAATTCTAATAGTTTGTTCCGACCTTCCACTGTGGACTCAATAGCAGG-GAGATGAAGAGGACAGTGATTGCATGA
hsa-miR-671-5p	AGGAAGCCCUGGAGGGGCUGGAG
hcmv-miR-US4	CGACAUGGACGUGCAGGGGGAU
hsa-miR-1285	UCUGGGCAACAAAGUGAGACCU
hsa-miR-455-3p	GCAGUCCAUGGGCAUAUACAC
58SrRNA	CGACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGAACGCAGCTAGCTGCGA-GAATTAATGTGAATTGCAGGACACATTGATCATCGACACTTCGAACGCACTTGCGGCCCCGGGTTCCTCCCGGGGCTACGCCTGTCTGAGCG

Using high throughput small non-coding RNA datasets, we continued to search for candidate mTBI biomarkers that are differentially regulated in PBMCs of mTBI compared to non-mTBI cases. Two criteria were used to identify candidate small RNA biomarkers for mTBI: 1) group changes (mTBI vs. non-mTBI groups) must be associated with a magnitude of $\geq$ 1.5-fold, and 2) group changes must be statiscally significant with $p < 0.05$, based on t-test analysis followed by the application false discovery rate corrections for multi-sampling errors. We identified 18 candidate small RNA biomarkers meeting both criteria: 4 miRNAs, 13 snoRNAs and 1 small scaRNA. Interestingly, we observed that all candidate small RNA biomarkers are significantly down-regulated in mTBI versus non-mTBI cases. In an unsupervised clustering analysis using RNA expression data generated from the high-throughput gene chip platform for the 18 candidate small RNA biomarkers, we were able to correctly segregate all 17 mTBI and non-mTBI cases analyzed (**Figure 2**), implicating their potential value as surrogate biological indices for mTBI among the OEF/OIF Veteran population.

Independent qPCR validation of candidate small non-coding RNA biomarkers

We next used an independent quantitative real-

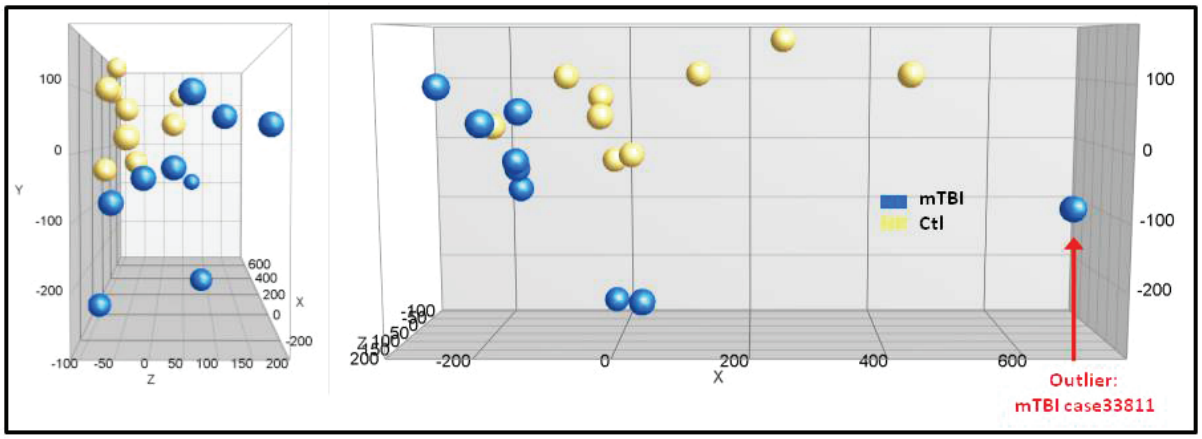

Figure 1. Principal component analysis of PBMC small RNAs from mTBI and non-mTBI veteran cases. Small RNA expression profiles for individual cases were assessed by a high-throughput Affymetrix Human gene 1.0 ST Array chip platform, which detected 428 small RNAs from PBMC specimens. Signals from all small RNA detected for each of the 9 mTBI and 9 non-mTBI cases were summarized into single points (represented by balls) plotted on a 3-dimensional plot. Blue and yellow balls represent, respectively, mTBI and non-mTBI cases. The analysis revealed that all cases can be clustered into an mTBI or a non-mTBI group, with the exception of mTBI case #33811 (indicated by a red arrow), which is plotted far away from the mTBI cluster.

time polymerase chain reaction (qPCR) procedure to assess the expression of individual candidate small RNA biomarkers in PBMC specimens from the same 9 mTBI and 9 non-mTBI cases used in our high-throughput biomarker discovery studies. Primer sets specific for each of the biomarkers were custom-designed and synthesized commercially by Applied Biosystems (Carlsbad, CA). Using these primer sets, we conducted qPCR studies and assessed the contents of individual candidate small RNA biomarkers in PBMCs of mTBI compared to non-mTBI cases. Results from our qPCR studies confirmed that 13 of the 18 candidate small RNA biomarkers are, indeed, differentially regulated in PBMCs of mTBI compared to non-mTBI veteran cases (**Figure 3**). The 13 confirmed small RNA biomarkers include 12 small nucleolar RNA (ACA48, ENSG199411, HBII-239, HBII-289, U15B, U27, U35A, U55, U56, U58B, U83A, U91) and 1 miRNA (Has-miR-671-5p) (**Figure 3**). Consistent with evidence from our RNA array platform, each of the 13 confirmed small RNA biomarkers are found in significantly lower levels in PBMC specimens from mTBI compared to non-mTBI veteran cases (**Figure 3**).

Exploring the potential value of novel small RNA biomarkers for segregating mTBI from non-mTBI veteran cases

Based on results from our qPCR biomarker confirmation studies, we next assessed the value of the 13 confirmed small RNA biomarkers as a criterion to correctly diagnose mTBI versus non-mTBI veteran cases. Using an unsupervised clustering analysis, we found the 13 confirmed mTBI biomarkers effectively segregated the cases correctly into mTBI and non-mTBI groups, with the exception of 2 of the non-mTBI cases which were incorrectly identified as mTBI (**Figure 4A**). We continued unsupervised clustering analyses to test the efficacy of individual or a combination of qPCR confirmed biomarkers to correctly segregate mTBI and non-mTBI veteran cases. Outcomes from these analyses led to the identification of a 3 small nucleolar biomarker panel, comprised of HBII-289, ENSG199411 and U35A, which is capable of distinguishing mTBI from non-mTBI veteran cases with 89% accuracy, 82% selectivity and 78% specificity (**Figure 4B**).

Discussion

Evidence has suggested that appropriate interventions can reduce functional impairment after mTBI [30,31,32,33]. In order to demonstrate the efficacy of clinical interventions, research must identify the biological, clinical, and neurological indices that are sensitive to the detection of functional impairments after mTBI. Results from our studies led to the identification of 13 novel clinically accessible small RNA mTBI

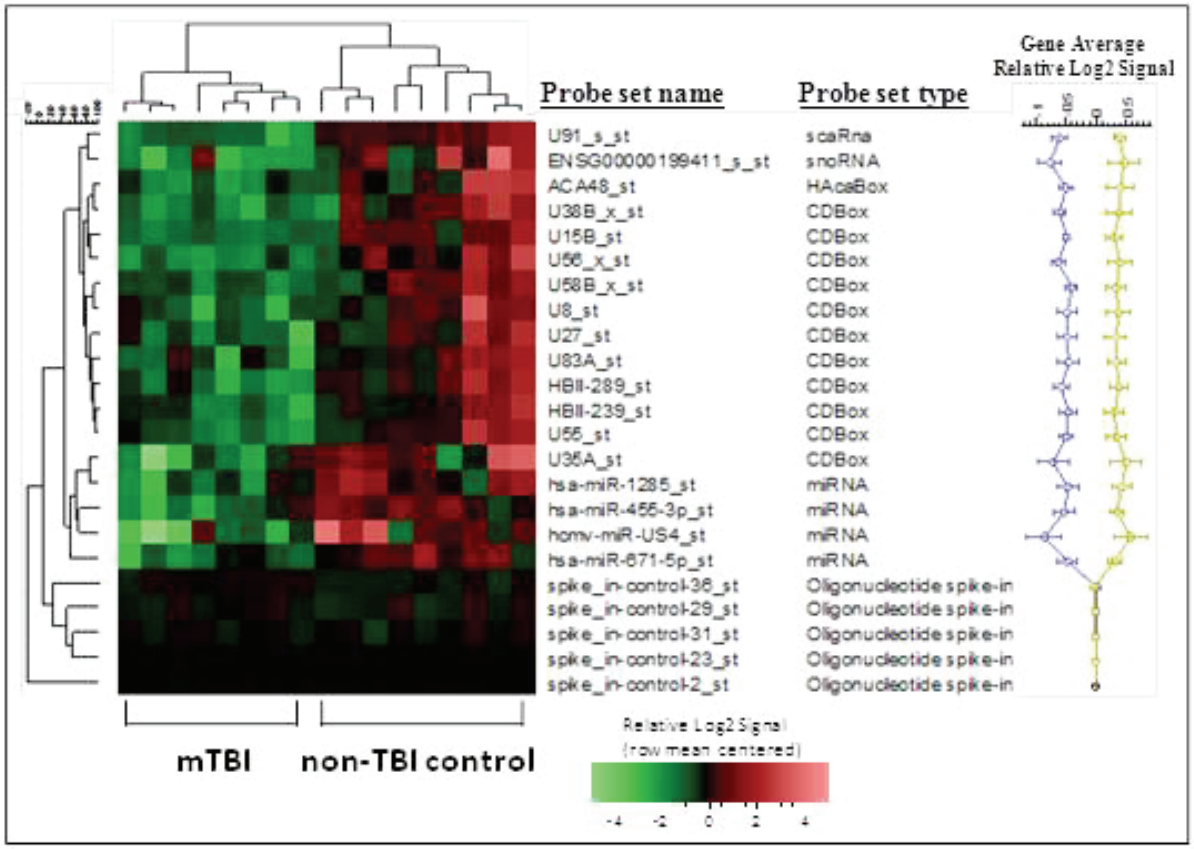

Figure 2. Unsupervised clustering analysis of 18 candidate small RNA TBI biomarker species. The 18 differentially-regulated small RNAs identified from interim high-throughput Array Chip analysis of 8 mTBI and 9 non-mTBI cases were subjected to unsupervised hierarchical clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are presented as a heat map (left panel) demonstrating that the panel of 18 small RNA biomarker species is able to correctly segregate mTBI from non-mTBI cases. Names for each of the small RNA biomarker species are identified under "Probe Set Name". Small RNA classes (and subclasses) that these 18 differentially-regulated mTBI biomarkers belong to are shown under "Probe Set Type". Vertical dendrogram (right panel) presents average (+/- SD) signal detections from mTBI versus non-mTBI groups for each of the 18 candidate small RNA biomarkers and confirmed divergent regulations of the biomarkers in PBMC specimens from mTBI vs. non-mTBI groups. Differential regulations of the 18 candidate biomarkers likely reflect true biological effects and not systematic experimental artifact(s) since there are no observable group differences for the detection of spike-in control oligonucleotides in all 17 OIF/OEF veteran cases analyzed (see heat map and vertical dendrogram). Abbreviations: miRNA, microRNA; snoRNA, small nucleolar RNA; C/D Box, the C/D box subclass of small nucleolar RNA; HAc Box, the HAc Box subclass of small nucleolar RNA; scaRNA, small Cajal body-specific RNA.

biomarkers, including 12 small snoRNA and 1 miRNA. Using qPCR, we have independently confirmed that each of these biomarkers is significantly down-regulated in PBMC specimens from mTBI compared to non-mTBI veteran cases. Among the 13 mTBI biomarkers, we demonstrated that a panel of 3 snoRNA - HBII-289, ENSG199411 and U35A – is capable of distinguishing mTBI from non-mTBI veterans with 89% accuracy, 82% selectivity and 78% specificity. Collectively, our evidence suggests that additional applications of the small RNA biomarkers we have identified, particularly the three biomarker panel, to current diagnostic criteria may improve mTBI detection and provide more sensitive outcome measures for clinical trials.

PTSD is commonly co-morbid with mTBI in OEF/OIF Veterans [34,35]. We note that the majority of Veterans with mTBI in our biomarker study have co-morbid PTSD and that our non-mTBI

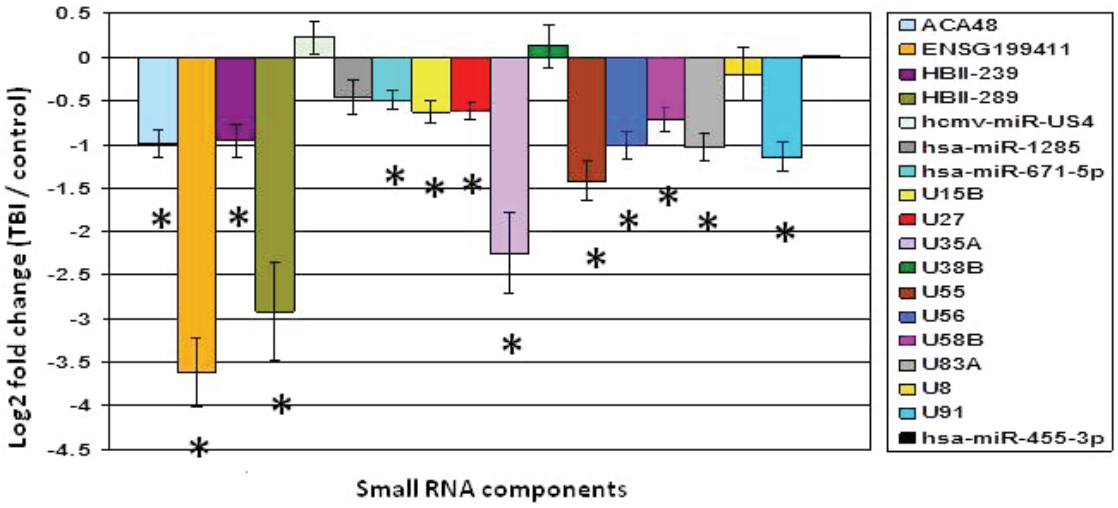

Figure 3. Independent quantitative real-time polymerase chain reaction (qPCR) assays confirmed that 13 small RNA TBI biomarkers are differentially regulated in PBMCs of mTBI relative to non-mTBI control cases. PBMC contents for each of the18 candidate small RNA biomarkers identified by the high-throughput Array Chip platform in Figure 2 were quantitatively assessed using independent qPCR assays. The same 9 mTBI and 9 non-mTBI cases (Table 1) we used in our initial high-throughput biomarker discovery assay were assessed in this qPCR biomarker confirmation study. Bar graphs represent mean small RNA biomarker contents in the mTBI group relative to the non-mTBI group; error bars represent standard errors. * False discovery rate-corrected P-value < 0.05. qPCR confirmed 13 small RNA biomarker species are significantly down-regulated in PBMC of mTBI compared to non-mTBI veteran cases. These 13 confirmed small RNA biomarkers include 12 small nucleolar RNA (ACA48, ENSG199411, HBII-239, HBII-289, U15B, U27, U35A, U55, U56, U58B, U83A, U91) and 1 miRNA (Has-miR-671-5p) species.

cases are selected to match for PTSD diagnosis. Thus, our identified 13 small RNA biomarkers likely represent biological indices selective for mTBI. Moreover, mTBI cases in our biomarker discovery studies were recruited after an average interval of 3.9 years following their last deployment (deployment-to-recruitment interval: ranging from 0.7 to 10.2 years, with a median interval of 3.4 years) (**Table 1**). Thus, changes in the regulation of these small RNA mTBI biomarkers that we observed are not acute mTBI responses, but likely represent long-term physiological consequences subsequent to mTBI experienced during deployment.

The pathological implication of our observation that select small nucleolar RNA and miRNA are differentially regulated in the PBMCs of mTBI relative to non-mTBI veteran cases is currently unknown. Small nucleolar RNA and miRNA are members of a family of non-coding RNAs that are involved in many physiological cellular processes and are also known to contribute to molecular alterations in pathological conditions [36]. SnoRNAs are short RNA sequences comprised of ~60-220 nucleotides. They are primarily known for their role as guide molecules for site-specific methylation and pseudouridylation of other RNAs, particularly rRNA, as well as tRNA and small nuclear RNAs. These chemical alterations are required for proper rRNA processing and ribosome function as well as for proper function of the spliceosome [37]. MicroRNA are short (~22 nucleotides) RNA sequences that bind to complementary sequences on target mRNA, thereby blocking translation or promoting degradation of target mRNA [37]. SnoRNA and miRNA are expressed in the brain and both classes of small RNAs have been implicated in neuroplasticity mechanisms and neurological disorders. For example, recent evidence suggests a role of the HBII52 small nucleolar RNA in the regulation of alternative splicing of the serotonin 2c receptor [38], and that patients with autism and Prader-Willi-like characteristics are found to have reduced levels of HBII52 in the brain [39]. The miRNA miR132 is induced by neuronal activity and neurotrophins in a CREB-dependent manner and plays a role in regulating neuronal morphology and cellular excitability [40]. Moreover, preclinical evidence in rodent models demonstrated that small RNA expression is affected in the brain by TBI. Redell et al. [8] reported transient elevated expression

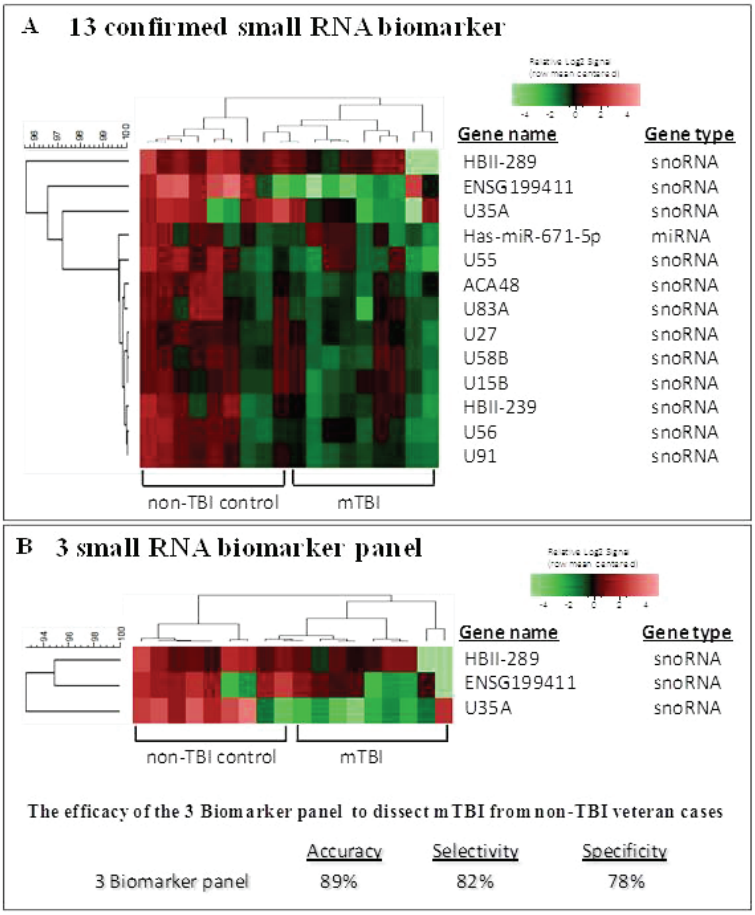

Figure 4. The content of small RNA biomarkers in clinically accessible PBMCs provides a sensitive and specific criterion for dissecting mTBI from non-mTBI veteran cases. We tested the role of the 13 qPCR confirmed small RNA mTBI biomarkers as a criterion for distinguishing mTBI from non-mTBI veteran cases. Biomarker contents in banked PBMC specimens from the same 9 mTBI and 9 non-mTBI cases (Table 1) were used in our biomarker discovery studies and quantified by qPCR. (A,B) The efficacy of using biomarker contents from clinically accessible PBMCs as a criterion to correctly segregate mTBI and non-mTBI cases was tested by unsupervised clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are presented as heat maps demonstrating the efficacy of using all 13 small RNA biomarkers (A) or using a panel of three small nucleolar RNA biomarkers (B) to correctly segregate mTBI from non-mTBI cases. (B) A three small nucleolar RNA biomarker panel (HBII-289, ENSG199411 and U35A) is capable of distinguishing mTBI from non-mTBI cases with 89% accuracy, 82% selectivity and 78% specificity (accuracy is the percentage of all mTBI and non-mTBI cases that are correctly identified; sensitivity is the probability that a case identified as mTBI actually is a mTBI case; specificity is the probability that a case identified as a non-mTBI case is actually a non-mTBI case). Abbreviations: snoRNA, small nucleolar RNA; miRNA, microRNA.

of the miRNA miRNA-21 in rats following an impact injury to the brain. Using a high-throughput Array Chip platform, Lei et al. [41] reported potential aberrant up- or down-expression of 203 miRNA species in the rat cerebral cortex up to 72 hrs following fluid percussion injury to the brain. Redell et al [42] also identified potential up-regulation of 35 and down-regulation of 50 miRNA species in the hippocampus of rats within 72 hrs following an impact TBI to the brain; altered regulations for a smaller subset of 8 (4 up-regulated and 4 down-regulated) miRNA species in the hippocampus were subsequently confirmed by independent qPCR.

It is possible that the altered regulation of select small nucleolar RNA and miRNA that we observed in PBMCs of veteran mTBI cases might have implications in the central nervous system. Genes relevant to neural circuits, synapses and neural plasticity processes are also expressed in circulating blood cells, such as PBMCs. Thus, the fact that we observed significant down-regulation of select small RNA biomarkers in PBMC specimens from our veteran mTBI cases long after their deployment might reflect long-term molecular alterations in the central nervous system contributing to the onset and progression of clinical TBI phenotypes. Future studies exploring the physiological relevance of mTBI biomarkers will provide a better understanding of the biological mechanisms underlying mTBI and insights into novel therapeutic targets for mTBI.

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Select small nucleolar RNAs in blood components as novel biomarkers for improved identification of comorbid traumatic brain injury and post-traumatic stress disorder in veterans of the conflicts in Afghanistan and Iraq

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ABSTRACT

The present study was designed to validate the ability of our recently identified set of small noncoding RNA candidate mild traumatic brain injury (mTBI) biomarkers to diagnose mTBI in the presence or absence of post-traumatic stress disorder (PTSD) comorbidity. Using qPCR, we explored the regulation of the candidate biomarkers in peripheral blood mononuclear cells (PBMC) from 58 veterans. We confirmed that 4 small nucleolar RNAs (snoRNAs), ACA48, U35, U55, and U83A, are significantly down-regulated in PBMC from veterans with mTBI and PTSD compared to non-TBI, control subjects with PTSD only. We found that the snoRNA biomarkers are able to dissect subjects with comorbid mTBI and PTSD from PTSD subjects without mTBI with 100% sensitivity, 81% accuracy, and 72% specificity. No significant differential expression of snoRNA biomarkers was found in mTBI subjects without comorbid PTSD. However, we found significantly lower U55 contents in subjects with PTSD. We explored the regulation of ACA48 in rodent models of PTSD or blast-induced mTBI to gather proof-of-concept evidence that would connect the regulation of the biomarkers and the development of mTBI or PTSD. We found no change in the regulation of ACA48 in the mTBI rat model. We did, however, find significant down-regulation of ACA48 in the PTSD mouse model 24 hours following psychological trauma exposure. This may reflect a short-term response to trauma exposure, since we found no change in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment. Additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD. Our observations suggest that biological interactions between TBI and PTSD may contribute to the clinical features of veterans with comorbid mTBI and PTSD. Future investigations on mTBI mechanisms or TBI biomarkers should consider their interactions with PTSD.

INTRODUCTION

Traumatic brain injury (TBI) has been referred to as the signature injury of veterans of the wars in Iraq and Afghanistan [1]. The majority of documented TBI cases among service members returning from Operation Enduring Freedom and Operation Iraqi Freedom (OEF/OIF) are characterized as mild-TBI (mTBI) [2]. Neurological and neuropsychiatric complaints, including mood changes and deficits in memory or attention, are very common in mTBI subjects. Current evidence suggests that oxidative stress and inflammatory responses following TBI play key roles in the accumulated cellular damage seen in TBI subjects [3]. Axonal damage due to mechanical shearing forces in the brain during the injury also contributes to the disruption in neuronal function and connectivity in the brains of TBI patients [4]. Accumulation of neuropathologic features associated with abnormal processing of the microtubule-associated protein, tau, may also contribute to long-term TBI complications [5].

TBI is associated with an increased risk for developing post-traumatic stress disorder (PTSD) [6,7], an anxiety disorder that may result from exposure to trauma. This may be due, in part, to an inability to suppress attention to trauma-related stimuli as a result of TBI-mediated neuronal damages [8]. Chronic inflammation in individuals that sustained a TBI may also contribute to PTSD [9,10]. The significant overlap of the symptoms associated with mTBI and comorbid PTSD complicates accurate mTBI evaluation and prognosis [6]. The current lack of easily accessible and accurate diagnostic biological fingerprints able to monitor mTBI clinical stages often impedes correct classification of mTBI/PTSD disease state, and thus the timely use of appropriate intervention. This has serious implications for veterans, since an estimated 7% (~119,720) of troops returning from Iraq and Afghanistan suffer from both TBI and PTSD [11]. Veterans with history of TBI and comorbid PTSD complain of more severe clinical complications, including neuropsychiatric symptoms (e.g., anxiety and depression) and neurocognitive dysfunctions, and therefore require more immediate intervention and support.

We previously reported that a set of thirteen small noncoding RNA candidate mTBI biomarkers have significantly lower levels of expression in accessible peripheral blood mononuclear cells (PBMC) from veterans with a history of mTBI compared to non-TBI control veterans [12]. However, the majority of mTBI and control subjects in our initial study had comorbid PTSD. Based on this consideration, the present studies were designed to validate, in a new veteran cohort, the ability of these candidate biomarkers to distinguish mTBI in the presence or absence of PTSD comorbidity.

MATERIALS AND METHODS

Study cohort: 58 OIF and OEF veteran cases (6 mTBI/PTSD, 11 non-TBI/PTSD, 7 mTBI/non-PTSD, and 34 non-TBI/non-PTSD) were recruited by The War Related Illness and Injury Study Center (WRIISC), Department of Veterans Affairs, New Jersey Health Care System (DVANJHCS), East Orange, NJ. Male and female participants were included if they were between 18-75 years of age and had completed a clinical evaluation at the New Jersey WRIISC. Participants were included regardless of their mTBI history. Cases with intercurrent infections or inflammatory-related conditions were excluded. Participants were classified as having a history of mTBI if they met at least one of 4 criteria on the veteran traumatic brain injury screening tool (VAT-BIST) [13], and had a score at least one standard deviation below the norm for age and education on the Repeatable Battery for Neuropsychological Testing (RBANS) [14]. Classification criteria for control cases included a negative VAT-BIST score and a RBANS score less than one standard deviation below the norm. Human participants were recruited by the War Related Illness and Injury Study Center following voluntary written informed consent. The study was approved by the Department of Veterans Affairs, New Jersey Health Care System Internal Review Board.

PBMC isolation: Blood specimens from human subjects were collected by venipuncture and drawn into BD Vacutainer CPT Cell Preparation Tubes (Becton, Dickinson and Company). PBMCs were isolated from freshly collected blood specimens according to the manufacturer's instructions, and were stored at -80 ^{o}C until use.

RNA preparation: Total RNA was isolated from approximately 10-50 mg of PBMCs using RNA STAT-60, according to the manufacturer's instructions (Tel-Test, Friendswood, TX, USA).

Quantitative Real-Time Polymerase Chain Reaction (qPCR) studies: Contents of targeted candidate small noncoding RNA biomarkers in PBMC specimens were assessed by qPCR using custom-designed qPCR primer sets, as we have previously done [12]. Data were normalized relative to those for the 5.8S ribosomal RNA. Levels of targeted small non-coding RNA were expressed relative to those in control groups using the $2^{-\Delta\Delta}$Ct method [15].

Biomarker assessments in experimental rodent models of mTBI or PTSD

Blast-induced rat model of mTBI: Adult male Long Evans hooded rats (250–350g; 10–12 weeks of age) were exposed to overpressure injury using the Walter Reed Army Institute of Research shock tube, which simulates the effects of air blast exposure under experimental conditions [16]. Rat blood specimens were collected 1.3 years following blast exposure via saphenous vein puncture. Parallel studies using age, gender, and strain-match rats without air blast exposure served as controls. All animal studies were approved by the James J. Peters Department of Veterans Affairs Medical Center (Bronx, NY), The Walter Reed Army Institute of Research and Naval Medical Research Center Institutional Animal Care and Use Committee, and the Icahn School of Medicine at Mount Sinai, (NY, New York) Institutional Animal Care and Use Committee and Institutional Review Board.

Chronic social defeat mouse model of PTSD: C57BL/6J mice were exposed to social defeat (SD) sessions once a day for 10 consecutive days, as previously described [17]. Twenty-four hours after the last (day 10) social defeat session, post-chronic social defeat blood specimens were collected by sub-mandibular bleeding. Parallel studies using age, gender, and strain-match animals not exposed to the social defeat protocol served as controls.

Assessments of small noncoding RNA biomarkers: Total RNA, including small RNA, were extracted from the blood using the PAXgene Blood miRNA Kit (PreAnalytiX, Qiagen), following the manufacturer's instructions. qPCR primers for mouse ACA48 and 5.8S rRNA were custom-designed and synthesized by Applied Biosystems. 10 ng of RNA was used in each reaction in

order to prepare cDNA, using the Taqman MicroRNA Reverse Transcription Kit. Data were normalized to those for 5.8S rRNA using the $2^{-\Delta\Delta Ct}$ method. Levels of target small non-coding RNA were expressed relative to those in the control groups.

RESULTS

Recruitment of 58 OEF/OIF cases for the biomarker study.

A total of 58 OIF/OEF veterans were recruited to test the value of our previously identified panel of 13 candidate biomarkers, either individually or in combination, to distinguish mTBI in the presence or absence of PTSD comorbidity. This study cohort is comprised of 13 mTBI cases and 45 non-TBI cases. The proportion of veterans (22%) classified with mTBI in our recruited cohort is consistent with, and even slightly above, previous prevalence estimates of 12% reported in a cross-sectional survey of 2,235 active duty, guard, and reserve OEF/OIF veterans [18]. Demographic information for the study cohort is shown in Table I. There were no significant differences between the mTBI and non-TBI groups with respect to group average values for age (31.2$\pm$8.6 and 34.8$\pm$10.3 years, respectively; p=0.25) or years of education (14.2±2.3 and 14.1±2.1 years, respectively; p=0.83). The mTBI group had a significantly longer time interval between their last deployment and their recruitment into this study (4.6±2.3 for the mTBI group and 2.8±2.3 years for the non-TBI group, p = 0.02). The proportion of males in the mTBI and the non-mTBI groups were 92% and 87%, respectively. Notably, our study cohort contained mTBI and non-TBI cases with or without co-morbid PTSD. This presents the opportunity to explore the ability of our previously identified candidate biomarkers to distinguish mTBI in the presence or absence of PTSD comorbidity.

PBMC expression of the candidate biomarkers in mTBI and control non-TBI cases comorbid with PTSD.

We previously identified 13 candidate small noncoding RNA mTBI biomarkers in an exploratory biomarker discovery study cohort of mTBI and non-TBI cases that were predominately comorbid with PTSD [12]. These 13 candidate small noncoding RNA mTBI biomarkers are listed in Fig. 1A. In this study, we continued to test for potential differentiation of these 13 small noncoding RNA biomarkers among mTBI cases with PTSD comorbidity, compared to control non-TBI

cases with PTSD (herein referred to as mTBI/PTSD and non-TBI/PTSD cases). Our current study cohort contained 6 mTBI/PTSD and 11 non-TBI/ PTSD cases (Table I). There were no significant differences between the mTBI/PTSD and non-TBI/PTSD groups with respect to group average values for age (31.5±10.7 and 33.1±11.08 years, respectively; p=0.67), post-deployment interval (5.3±2.5 and 3.6±2.5 years, respectively; p=0.20), or years of education (14.7±2.5 and 13.3±2.6 years; p=0.18). The proportion of males in the mTBI and the non-mTBI groups were 83% and 82%, respectively.

We quantified PBMC contents of individual candidate small noncoding RNA mTBI biomarkers in each of the cases, using qPCR. Consistent with observations from our initial exploratory biomarker discovery studies [12], we that found that 4 of the small noncoding RNA biomarkers, ACA48, U35A, U55, and U83A, are significantly down-regulated in mTBI/PTSD cases compared to non-TBI/PTSD cases (Fig. 1B). Our observations validate, for the first time, ACA48, U35A, U55, and U83A in PBMC as biomarkers of mTBI in the context of PTSD comorbidity. Moreover, using unsupervised hierarchical cluster analysis, we found that a combination of ACA48, U35A, U55, and U83A was able to correctly distinguish mTBI/PTSD cases from control non-TBI/PTSD cases with 82% accuracy, 100% sensitivity, and 72% specificity (Fig. 1C, D). The sensitivity and specificity of using the combined biomarker for correctly segregating mTBI/PTSD vs. non-TBI/PTSD cases in our present study cohort was confirmed using the receiver operating characteristic (ROC) analysis as an independent assessment.

We note that all 4 validated biomarkers of mTBI/PTSD comorbidity are members of the small nucleolar RNA (snoRNA) class that are known for their activities in modulating RNA splicing, stability, and/or translation [19–21]. Potential relevance of the 4 snoRNA biomarkers to TBI/PTSD pathophysiology will be discussed in more details in the *Discussion* section, below.

PBMC expression of ACA48, U35A, U55, and U83A in mTBI and control non-TBI cases without PTSD.

We continued to explore the regulation of the 4 snoRNA biomarkers in mTBI and non-TBI cases in the absence of PTSD. Our study cohort of 58 veteran cases contained 7 mTBI cases without PTSD and 34 non-TBI cases without PTSD (herein referred to as mTBI/non-PTSD and non-TBI/non-PTSD cases, respectively). Demographic information for the mTBI/non-PTSD and non-TBI/non-PTSD cases is shown in Table I. As a group, mTBI/non-PTSD cases were significantly younger than control non-TBI/non-PTSD cases (group averages 27.4±4.1 and 35.3±10.05 years, respectively; p-value 0.05). There were no significant differences between the mTBI/non-PTSD and non-TBI/non-PTSD groups with respect to group average values for post-deployment interval (3.9±2.1 and 2.5±2.2 years, respectively; p=0.15) or years of education (13.86±2.1 and 14.4±2.2 years, respectively; p=0.59). The proportion of males in the mTBI and the non-TBI groups were 100% and 88%, respectively.

We found no significant differences in PBMC contents of ACA48, U35A, U55, or U83A in mTBI vs. non-TBI cases in the absence of PTSD comorbidity (Fig. 2A). Moreover, we found that these 4 snoRNA biomarkers have no predictive value for segregating mTBI and non-TBI cases when they are not comorbid with PTSD (Fig. 2B, C, D).

PBMC expression of ACA48, U35A, U55, and U83A in PTSD vs. non-PTSD cases in the absence of TBI.

We also explored the potential effects of PTSD on regulation of the 4 validated snoRNA biomarkers. This study cohort contained 11 non-TBI/PTSD and 34 non-TBI/non-PTSD cases (Table I). There were no significant differences between the non-TBI/PTSD and non-TBI/non-PTSD groups with respect to group average values for age (33.1±11.1 and 35.4±10.1 years, respectively; p=0.53), post-deployment interval (3.6±2.5 and 2.6±2.2 years, respectively;

p=0.18), or years of education (13.3±1.6 and 14.4±2.2 years; p=0.14). The proportion of males in the non-TBI/PTSD and the non-TBI/non-PTSD group were 82% and 88%, respectively.

We found no significant differences in PBMC contents of ACA48, U35A, or U83A in PTSD vs. non-PTSD cases in the absence of mTBI comorbidity (Fig. 3A). Interestingly, we did find significant down-regulation of U55 in PBMC of non-TBI/PTSD compared to non-TBI/non-PTSD. This evidence suggests that PTSD has an effect on the regulation of U55, which may contribute to the down-regulation of U55 seen in mTBI vs. non-TBI cases in the context of PTSD comorbidity (Fig. 1A, B). Nonetheless, we found that application of the 4 validated mTBI/PTSD snoRNA biomarkers, ACA48, U35A, U55, and U83A, did not have any predictive value for differentiating PTSD from non-PTSD cases in the absence of mTBI comorbidity (Fig. 3B, C, D).

Regulation of the validated mTBI/PTSD snoRNA biomarkers in animal models of blast-induced mTBI or PTSD.

Recently, a blast-induced rat model mTBI was developed to demonstrate the effects of air blast exposure in OEF/OIF veterans [5]. Furthermore, mice exposed to chronic social defeat are being used to model anxiety and depression symptoms seen in PTSD [17,22]. Using the blast-induced rat mTBI model and the chronic social defeat mouse PTSD experimental model, we continued to explore potential cause-effect relationships between mTBI and/or PTSD and down-regulation of our validated snoRNA mTBI/PTSD biomarkers in PBMC. Among the 4 validated snoRNA mTBI/PTSD biomarkers of interest, rat sequence information is available only for ACA48. Therefore, we explored the regulation of ACA48 in rodent models of blast-induced mTBI or PTSD to gather proof-of-concept evidence that would show that mTBI and/or PTSD have implications on the regulation of the 4 snoRNA biomarkers.

We collected blood specimens from blast-treated rats ~1.3 years after their initial blast exposure to simulate the long-term post-deployment characteristics of the OEF/OIF cases in our initial biomarker discovery [12] and in our current biomarker validation studies. We found no change in the regulation of ACA48 in blood specimens from the blast-induced mTBI rat model compared to control rats (Fig. 4A), implicating that the down-regulation of ACA48 that we observed in veterans with comorbid mTBI and PTSD (Fig. 1A,B) might not be due to exposure to blast-induced mTBI. Further experimental assessments evaluating the potential impact of blast exposure on the regulation of U35A, U55, and U83A in the blast-induced mTBI rat model will have to wait for the availability of rat sequence information for these snoRNA biomarkers.

Exploring the potential influence of PTSD on the regulation of ACA48, we observed a significant down-regulation of ACA48 (by approximately 50%) in blood cells of mice exposed to chronic social defeat (SD) compared to control mice (Fig.4B). Our observation demonstrates, for the first time, that experimental PTSD is associated with altered regulation of a specific snoRNA, implicating that the presence of PTSD may contribute to the observed down-regulation of ACA48 in veterans with comorbid mTBI and PTSD. We note that our experimental evidence of down-regulation of ACA48 was based on monitoring the PTSD mouse model within a short time frame (by 24 hours) after completion of the chronic social defeat protocol. Interestingly, we found no observable changes in the content of ACA48 in PTSD vs. non-PTSD cases in the absence of TBI long after the veterans' deployment (average post deployment interval of 3.6 years) (Fig. 3A), suggesting that while PTSD may have a direct acute impact on the regulation of ACA48 in PBMC, this effect may not last over the long-term. Additional studies will be required to test longer-term impacts of PTSD on the regulation of ACA48, U35A, U55, and U83A in this PTSD mouse model.

DISCUSSION

Validation of select snoRNA biomarkers for distinguishing veteran mTBI cases with or without PTSD comorbidity.

We confirmed that 4 snoRNA species, ACA48, U35A, U55, and U83A, are significantly down-regulated in PBMC specimens from mTBI/PTSD compared to control, non-TBI/PTSD cases in two independent study cohorts. Moreover, we established the ability of these 4 validated biomarkers to distinguish cases with or without mTBI in the context with PTSD comorbidity. Identification of these snoRNA biomarkers will provide for improved detection of co-morbid mTBI and PTSD and for more sensitive measurements for clinical trials.

We observed that the 4 validated snoRNA mTBI/PTSD biomarkers were not differentially regulated among mTBI and non-TBI in the absence of PTSD comorbidity, suggesting that the presence of mTBI alone is insufficient to induce the down-regulation of ACA48, U35A, U55, and U83A seen in cases of comorbid mTBI and PTSD. Consistent with this, our ongoing studies observed no detectable change in contents of ACA48 in blood cells from a blast-induced TBI rat model.

We found significantly lower contents of U55 in PBMC from non-TBI/PTSD vs. non-TBI/non-PTSD, but no detectable changes in the regulation of ACA48, U35A, or U83A in PBMC of OEF/OIF veterans with PTSD compared to veterans without PTSD in the absence of TBI comorbidity long after their deployment. Interestingly, our ongoing studies using the chronic social defeat PTSD mouse model suggest that PTSD may lead to down-regulation of ACA48 over the short-term. This may reflect a short-term, acute response to trauma exposure, since we found no changes in the regulation of ACA48 in veteran PTSD subjects 3.6 years post-deployment. Additional investigations will be necessary to explore the short- and long-term

contributions of PTSD on the down regulation of ACA48, U35A, U55, and U83A in veterans with comorbid mTBI and PTSD.

Our evidence suggests that the presence of mTBI alone is insufficient to induce down-regulation of these snoRNA biomarkers, but the presence of PTSD may contribute to the down-regulation of U55 and ACA48 seen in mTBI veterans with comorbid mTBI and PTSD. Our observations suggest that biological interactions between TBI and PTSD may contribute to the clinical features of mTBI with comorbid PTSD.

Most mTBI cases among veterans of the conflicts in Afghanistan and Iraq are due to blast-induced injuries. However, we do not have any information on our study cohort in regards to the type of injury—blast versus non-blast—that individual cases were exposed to. Future studies will be necessary to explore the impact of blast and non-blast TBI injuries on the regulation of these snoRNA biomarkers.

Potential relevance of the four snoRNA biomarkers to TBI/PTSD pathophysiology.

The 4 small noncoding RNA mTBI/PTSD biomarkers that we validated in our present studies are all members of the small nucleolar RNA (snoRNA) class. SnoRNAs are known for their methylation and pseudouridylation of other small noncoding RNAs, particularly ribosomal RNAs, transfer RNAs, and small nuclear RNAs [23]. Methylation/pseudouridylation of small noncoding RNAs may modulate the folding of small noncoding RNAs and protect small noncoding RNAs from hydrolysis [19]. Recent evidence demonstrates that select snoRNA (snoRNA HBII-52) is also involved in alternative splicing of the serotonin receptor 2C [20]. Changes in the regulation of snoRNAs have been associated with multiple cancers, and deletion of imprinted snoRNAs in chromosome 15q11-q13 has been associated with Prader-Willi syndrome [21]. There is, however, little information on the potential biological activities of ACA48, U35A, U55, and U83A.

Recent evidence revealed that certain snoRNAs, referred to as sno-miRNAs, may also exhibit microRNA (miRNA) functions by interfering with translation and/or by promoting degradation of targeted mRNAs [24]. Interestingly, one of our snoRNA biomarkers, U83A, is among the sno-miRNAs reported to exert sno-miRNA activities [24]. Ongoing studies are attempting to characterize specific gene targets of U83A.

Another snoRNA biomarker validated in our present study, U35A, has been shown to be effective in modulating cellular responses to oxidative stress and inflammatory mediators. It has also been demonstrated that knockdown of U35A mitigates lipopolysaccharides-induced oxidative stress in the liver [25]. While the mechanisms of action are presently unknown, these observations suggest that down-regulation of U35A in PBMC of veterans with comorbid TBI and PTSD may reduce resilience to inflammatory stress, leading to the promotion of oxidative stress conditions and contributing to mTBI and/or PTSD clinical features over the long-term.

CONCLUSIONS

Collectively, evidence from our studies suggest that additional application of the 4 snoRNA biomarker to current diagnostic criteria may provide an objective biomarker pattern to help identify veterans with comorbid mTBI and PTSD. Furthermore, future studies clarifying the potential bioactivity of ACA48, U55, and U83A will provide additional insight on pathogenic mechanisms contributing to long-term clinical complications in veterans with comorbid TBI/PTSD. Moreover, observations from our studies suggest that future investigations on mTBI biomarkers, as well as pathogenic mechanisms underlying mTBI, should carefully consider the impact of interactions with PTSD.

LIST OF ABBREVIATIONS USED

Afr Am, African American

AUG, area under the curve

Bl, black

cDNA, complementary DNA

DVANJHCS, Department of Veterans Affairs, Bew Jersey Health Care System

FP, false positive

Hisp, hispanic

mTBI, mild traumatic brain injury

nHisp, non-hispanic

OEF, Operation Enduring Freedom

OIF, Operation Iraqi Freedom

PTSD, post-traumatic stress disorder

PBMC, peripheral blood mononuclear cells

qPCR, real-time quantitative polymerase chain reaction

RBANS, Repeatable Battery for Neuropsychological Testing

ROC, Receiver Operating Characteristic

snoRNP, small nucleuolar ribonucleoprotein

snoRNA, small nucleolar RNA

TBI, traumatic brain injury

TP, true positive

VA-BIST, Veteran Traumatic brain injury screening tool

Wh, white

WRIIC, War Related Illness and Injury Study Center

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The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. Some of the authors are military service member (or employees of the U.S. Government). This work was prepared as part of their official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

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TABLE LEGEND

Table I: Demographic characteristics of the study cohort. Information is presented for the 58 cases recruited for this study. Cases are subgrouped by characterization of mTBI with comorbid PTSD, non-TBI with PTSD, non-TBI without PTSD, and non-TBI without PTSD. Abbreviations; Wh, white; Hisp, hispanic; nHisp, non-hispanic; Bl, black; Afr Am, African American.

FIGURE LEGEND

Figure 1: Candidate mTBI small noncoding RNA biomarker expression in TBI vs non-TBI cases in the context of PTSD comorbidity. (A, B) Contents of candidate small noncoding RNA biomarkers in PBMC from mTBI/non-PTSD and non-TBI/non-PTSD cases were assessed by qPCR. A) t-test p-value comparisons between mTBI/PTSD vs. non-TBI/PTSD cases. Red letters highlight candidate small noncoding RNA mTBI biomarkers with significant differential regulation in PBMC specimens of mTBI/PTSD compared to non-TBI/PTSD cases. B) Bar graphs show mean±SEM fold change (in Log2 values) of candidate mTBI biomarkers in mTBI/PTSD vs. control non-TBI/PTSD cases. Values <0 or >0 indicate, respectively, down-regulation or up-regulation in mTBI/PTSD compared to non-TBI/PTSD cases. Arrows point to the 4 small noncoding RNAs that were significantly down-regulated (* $p<0.05$) in mTBI/PTSD vs. control non-TBI/PTSD cases. (C, D, E) The combination of a 4-biomarker panel, ACA48, U35A, U55, and U83A, provides a sensitive and specific criterion for differentiating mTBI/PTSD from non-TBI/PTSD cases. Unsupervised hierarchical clustering of mTBI/PTSD and non-TBI/PTSD cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples into one of two major clusters. One of the major clusters contained a majority of the mTBI samples and was designated as the mTBI cluster, while the other major cluster was designated as the non-TBI cluster. A) Summation table of the unsupervised hierarchical clustering analyses using combinations of the 4 biomarkers, ACA48, U35A, U55, and U83A, to correctly identify mTBI/PTSD vs. non-TBI/PTSD cases. Accuracy represents the percentage of all mTBI/PTSD and non-mTBI/PTSD cases that were correctly diagnosed by the test, calculated as the number of correctly identified mTBI/PTSD and non-TBI/PTSD cases divided by the total number of cases analyzed. Sensitivity (true positive [TP]/[TP + false negative (FN)]) is the probability that a case predicted to have mTBI actually had it, whereas specificity (true negative [TN]/[false positive (FP) + TN]) measures the probability that a case predicted not to have mTBI did, in fact,

not have it. (B) Heat map of an unsupervised hierarchical clustering analysis using the combined 4 small noncoding RNA biomarkers (ACA48, U35A, U55, and U83A) showing segregation of mTBI/PTSD and non-TBI/PTSD cases. C) Receiver Operating Characteristic (ROC) analysis using the combined 4 biomarkers. ROC curve plotting the percentage of correctly identified mTBI/PTSD cases (true positive; specificity) as a function of the percentage of non-TBI/PTSD cases incorrectly identified as mTBI/PTSD cases (false positive; 1-specificity). The calculated area under the curve (AUC) is 1.0. In general, an AUC of 1 indicates the capability of a test to perfectly segregate two populations, and an AUC of 0.5 indicates that the test cannot segregate two populations beyond chance. This evidence supports the hypothesis that the combined 4 small noncoding RNA biomarker is a vigorous and sensitive test to distinguish mTBI/PTSD from non-mTBI/PTSD cases.

Figure 2. Candidate mTBI snoRNA biomarker expression in mTBI vs. non-TBI control cases in the absence of PTSD comorbidity. Contents of ACA48, U35A, U55, and U83A in PBMC from mTBI/non-PTSD and non-TBI/non-PTSD cases were assessed by qPCR. A) t-test p-value comparison between mTBI/non-PTSD and non-TBI/non-PTSD cases. B) Unsupervised hierarchical clustering of mTBI/non-PTSD and non-TBI/non-PTSD cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples into either the mTBI/non-PTSD or the non-TBI/non-PTSD cluster. Presented is a summation table of unsupervised hierarchical clustering analyses using combinations of ACA48, U35A, U55, and U83A to correctly distinguish mTBI/non-PTSD vs. non-TBI/non-PTSD cases. (C) Heat map of an unsupervised hierarchical clustering analysis using the combined 4 snoRNA biomarkers demonstrates a lack of effective segregation between mTBI/PTSD and non-TBI/PTSD cases. D) ROC analysis using the combined 4 biomarkers. ROC curve plotting the percentage of correctly identified mTBI/non-PTSD cases (true positive; specificity) as a function of the percentage of non-TBI/non-PTSD

cases incorrectly identified as mTBI/non-PTSD cases (false positive; 1-specificity). The calculated area under the curve (AUC) is 0.64.

Figure 3. Candidate mTBI snoRNA biomarker expression in PTSD vs. non-PTSD control cases in the absence of TBI comorbidity.

A) PBMC contents of ACA48, U35A, U55, and U83A in PTSD/non-TBI and non-PTSD/non-TBI cases, as assessed by qPCR. Bar graphs present mean and SEM values; * $p<0.05$, t-test p-value comparison between PTSD/non-TBI vs. non-PTSD/non-TBI cases. B,C) Unsupervised hierarchical clustering of PTSD/non-TBI and non-PTSD/non-TBI cases was conducted using the Unweighted Pair Group Method with Arithmetic Mean agglomerative method. Cluster assignment was based on assigning samples to PTSD/non-TBI and non-PTSD/non-TBI clusters. Presented is a summation table of unsupervised hierarchical clustering analyses using combinations of ACA48, U35A, U55, and U83A to correctly identify PTSD/non-TBI and non-PTSD/non-TBI cases (B). Heat map of an unsupervised hierarchical clustering analysis shows that a combination of ACA48, U35A, U55, and U83A is not effective in segregating PTSD/non-TBI vs. non-PTSD/non-TBI cases (C). D) ROC analysis using ACA48, U35A, U55, and U83A. The calculated area under the curve is 0.75.

Figure 4. ACA48 snoRNA expression in blood cells from experimental rodent models of PTSD and TBI.

A blast-induced mTBI rat model [5] and a chronic social defeat (SD) mouse model of PTSD [17]) were used in these studies. Contents of ACA48 snoRNA biomarker in blood cells were analyzed by qPCR using primer sets designed for targeting rat or mouse ACA48. A) Expression of ACA48 in blood cells from rats exposed to blast-mediated TBI rats compared to control rats. Blood specimens were collected ~1.3 years after initial blast exposure. B) Expression of ACA48 in blood cells from mice subjected to SD compared to control mice. Blood specimens were

collected within a short time (~24 h) after completion of the chronic SD protocol. (A, B) Bar graphs represent ACA48 content normalized to those for 5.8S rRNA using the $2^{-\Delta\Delta Ct}$ method. Levels of target small non-coding RNA were expressed relative to those in the control groups. T-test, * $p<0.05$.

Table I:

Case #	mTBI/non-TBI	age	sex	Ethnicity	Interval (yrs) since last deployment	Education (yrs)	PTSD Comorbidity
33361	mTBI	26	F	Wh/Hisp	3.2	13	Yes
34084	mTBI	32	M	Bl/Afr Am/nHisp	3.5	13	Yes
34119	mTBI	29	M	Wh/nHisp	8.1	18	Yes
34124	mTBI	47	M	Wh/nHisp	2.6	18	Yes
34193	mTBI	51	M	Wh/nHisp	6.7	13	Yes
34260	mTBI	28	M	Bl/Afr Am/nHisp	7.9	13	Yes
33478	mTBI	30	M	Wh/nHisp	6.4	18	No
33570	mTBI	23	M	Wh/Hisp	2.8	13	No
34055	mTBI	28	M	Wh/Hisp	3.2	14	No
34128	mTBI	24	M	Wh/nHisp	1.5	12	No
34138	mTBI	26	M	Wh/nHisp	2.6	12	No
34254	mTBI	35	M	Wh/nHisp	7.2	15	No
34261	mTBI	26	M	Wh/nHisp	3.7	13	No
33759	non-TBI	28	M	Wh/Hisp	0.4	12	Yes
33899	non-TBI	60	M	Wh/nHisp	not deployed	14	Yes
33913	non-TBI	22	F	Bl/Afr Am/nHisp	1.0	12	Yes
33944	non-TBI	30	M	Wh/nHisp	8.4	14	Yes
33848	non-TBI	25	M	Bl/Afr Am/nHisp	4.3	13	Yes
33977	non-TBI	42	M	Bl/Afr Am/nHisp	2.3	14	Yes
34060	non-TBI	27	F	Wh/nHisp	3.9	17	Yes
34070	non-TBI	24	M	Wh/Hisp	1.2	12	Yes
34079	non-TBI	37	M	Wh/nHisp	4.3	14	Yes
34087	non-TBI	40	M	Wh/Hisp	4.4	12	Yes
34160	non-TBI	29	M	Wh/nHisp	6.0	12	Yes
30597	non-TBI	40	M	Wh/nHisp	4.4	18	No
31429	non-TBI	39	M	Bl/Afr Am/nHisp	0.9	14	No
31824	non-TBI	43	M	Bl/Afr Am/nHisp	6.5	13	No
33673	non-TBI	25	M	Wh/Hisp	2.8	14	No
33676	non-TBI	32	M	Wh/Hisp	1.0	16	No
33699	non-TBI	42	M	Bl/Afr Am/nHisp	0.7	16	No
33765	non-TBI	26	F	Wh/Hisp	0.9	16	No
33786	non-TBI	52	M	Bl/Afr Am/nHisp	0.7	14	No
33819	non-TBI	24	M	Wh/Hisp	1.9	12	No
33832	non-TBI	43	M	Wh/nHisp	4.4	12	No
33858	non-TBI	29	M	Bl/Afr Am/nHisp	6.5	18	No
33863	non-TBI	41	M	Wh/nHisp	0.8	12	No
33864	non-TBI	23	M	Wh/Hisp	1.8	14	No
33877	non-TBI	59	M	Wh/nHisp	0.7	16	No
33904	non-TBI	22	M	Asian	0.6	12	No
33905	non-TBI	38	M	Wh/nHisp	1.1	18	No
33911	non-TBI	29	M	Bl/Afr Am/nHisp	7.7	16	No
33915	non-TBI	25	F	Bl/Afr Am/nHisp	4.2	14	No
33918	non-TBI	33	M	Wh/Hisp	1.7	12	No
33958	non-TBI	23	M	Wh/Hisp	1.2	12	No
33992	non-TBI	28	M	Wh/nHisp	4.7	13	No
34026	non-TBI	34	M	Asian	1.3	13	No
34047	non-TBI	45	F	Bl/Afr Am/nHisp	4.9	15	No
34076	non-TBI	40	M	Bl/Afr Am/nHisp	0.7	12	No
34077	non-TBI	25	M	Wh/Hisp	1.2	13	No
34093	non-TBI	47	M	Wh/nHisp	1.9	16	No
34094	non-TBI	42	M	Wh/nHisp	3.8	17	No
34117	non-TBI	42	M	Wh/nHisp	0.4	20	No
34120	non-TBI	54	M	Wh/nHisp	5.2	16	No
34126	non-TBI	34	M	Wh/Hisp	6.8	13	No
34142	non-TBI	46	M	Wh/Hisp	1.7	14	No
34192	non-TBI	24	M	Wh/Hisp	1.6	12	No
34247	non-TBI	26	F	Wh/Hisp	1.0	13	No
33856	non-TBI	27	M	Wh/Hisp	0.6	12	No

Figure
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Figure 1:

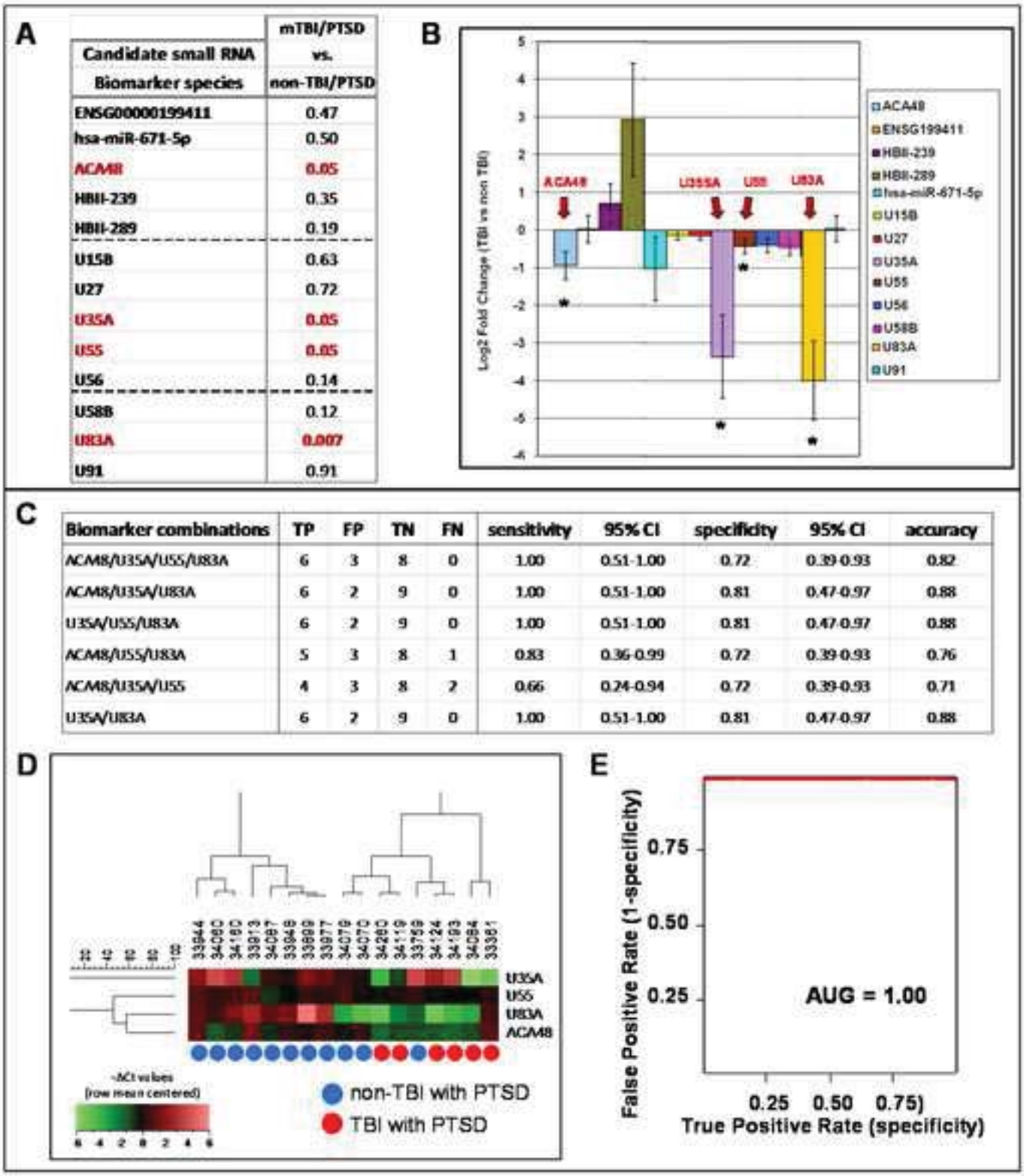

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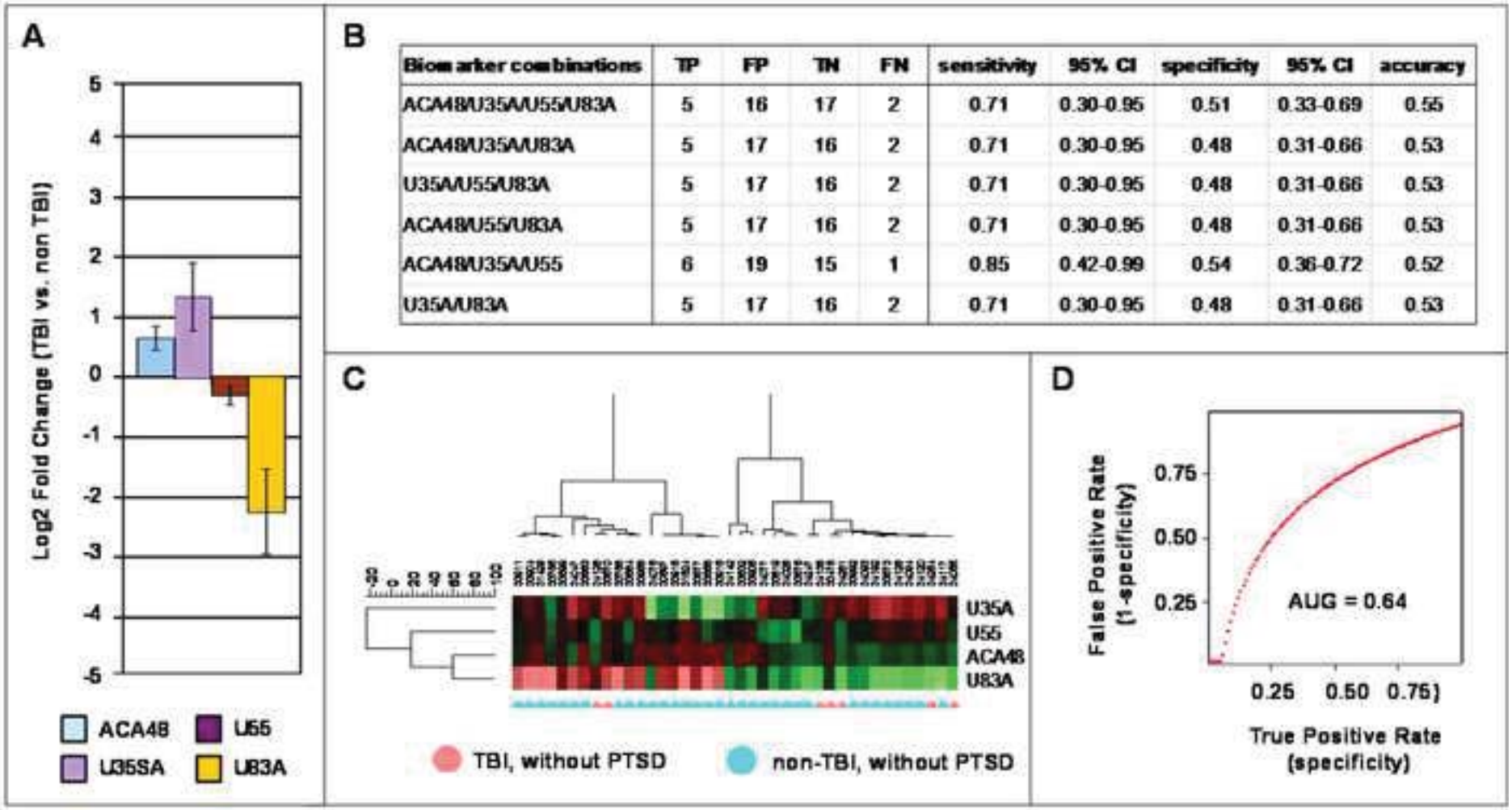
Figure 2:



Biomarker combinations	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	5	16	17	2	0.71	0.30-0.95	0.51	0.33-0.69	0.55
ACA48/U35A/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
U35A/U55/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
ACA48/U55/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53
ACA48/U35A/U55	6	19	15	1	0.85	0.42-0.99	0.54	0.36-0.72	0.52
U35A/U83A	5	17	16	2	0.71	0.30-0.95	0.48	0.31-0.66	0.53

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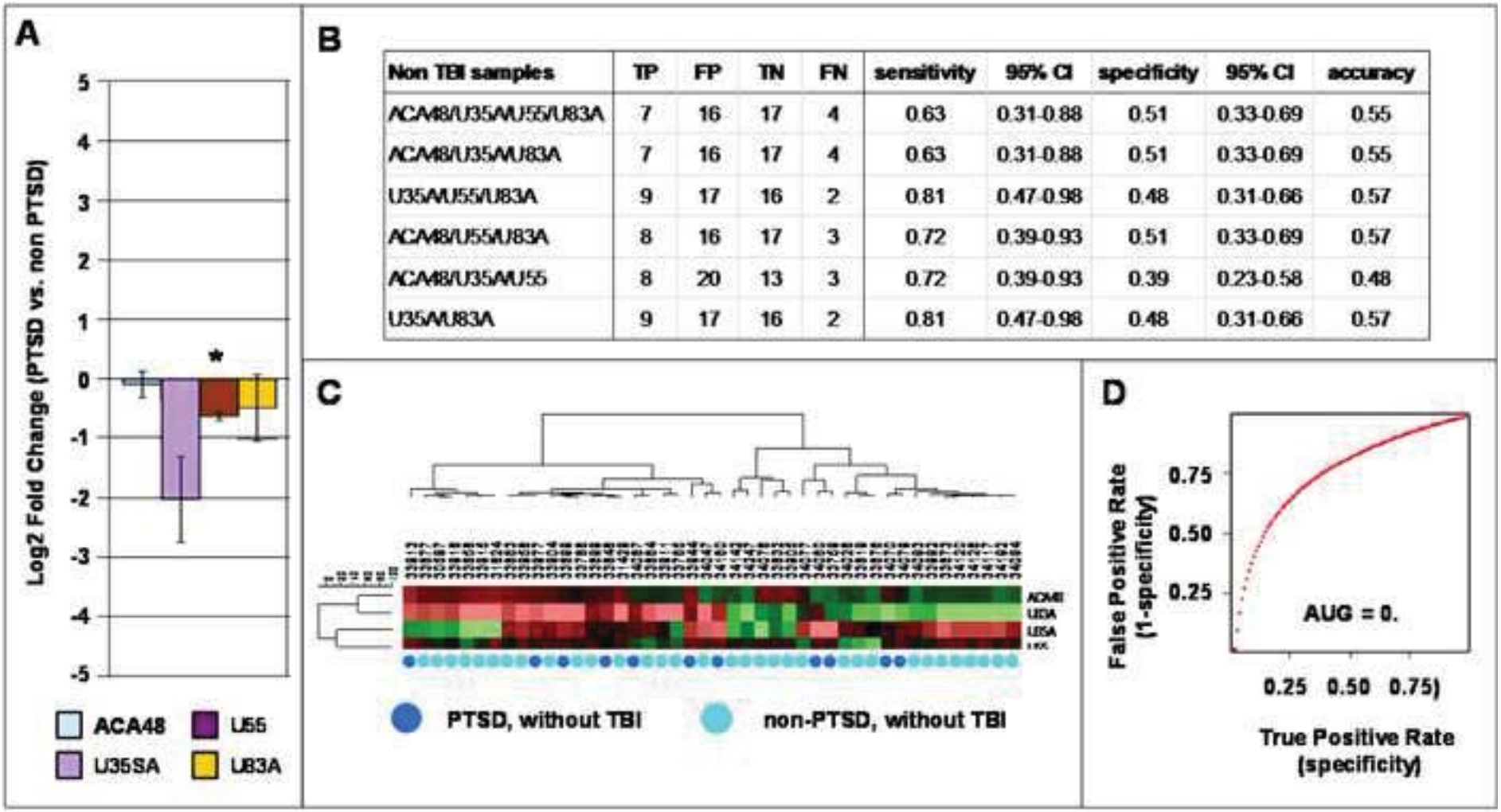
Figure 3:



Non TBI samples	TP	FP	TN	FN	sensitivity	95% CI	specificity	95% CI	accuracy
ACA48/U35A/U55/U83A	7	16	17	4	0.63	0.31-0.88	0.51	0.33-0.69	0.55
ACA48/U35A/U83A	7	16	17	4	0.63	0.31-0.88	0.51	0.33-0.69	0.55
U35A/U55/U83A	9	17	16	2	0.81	0.47-0.98	0.48	0.31-0.66	0.57
ACA48/U55/U83A	8	16	17	3	0.72	0.39-0.93	0.51	0.33-0.69	0.57
ACA48/U35A/U55	8	20	13	3	0.72	0.39-0.93	0.39	0.23-0.58	0.48
U35A/U83A	9	17	16	2	0.81	0.47-0.98	0.48	0.31-0.66	0.57

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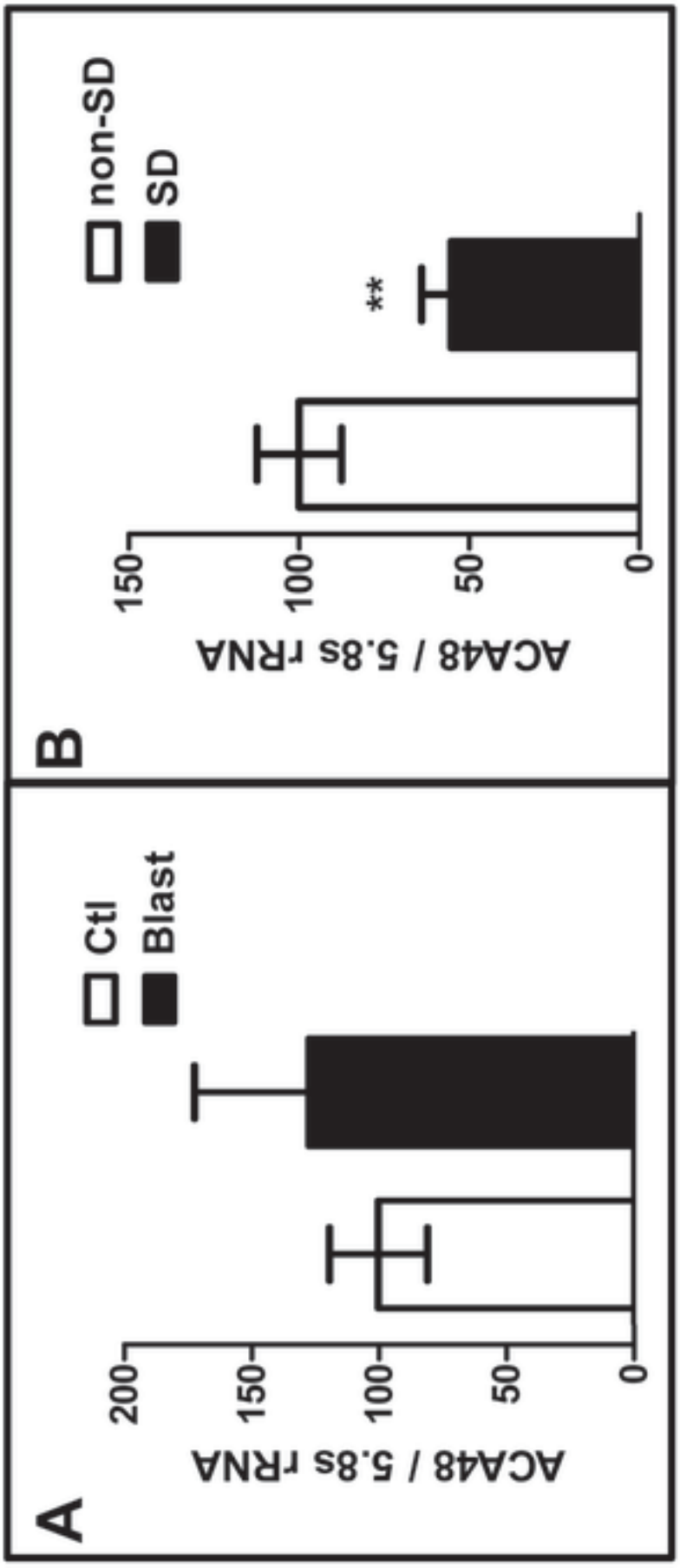

Figure 4: